The role of p21 in regulation of injury response in lung epithelial cells

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Declaration.

I declare that the work presented in this thesis is original and is not copied from other sources. References to other works are well documented.

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List of abbreviations

Ab	antibody
ABC	avidin-biotin complex
ABC-AP	avidin-biotin complex-alkaline phosphatase
APC	adenomatous polyposis cell
ARF	alternative reading frame
ASK	apoptosis signal-regulating kinase 1
APUD	amine precursor uptake and decarboxylation
BAL	bronchoalveolar lavage
Bax	Bcl-associated X protein
Bcl-2	B-cell lymphoma
bp	base pair
BPA	*Bauhinia purpurea*
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CAS	cadherin associated substrate
CC10	Clara cell 10kDa protein
CC16	Clara cell 16kDa protein
CCSP	Clara cell secretory protein
cDNA	complementary DNA
Cdk	cyclin-dependent kinase
CDKI	cyclin dependent kinase inhibitor
CEBPα	CCAAT/enhancer binding protein α
CFA	cryptogenic fibrosing alveolitis
CFTR	cystic fibrosis transmembrane regulator
CIP	Cdk-inhibitory protein
CKI	cyclin kinase inhibitors
Cl^-	chloride ion

Coll IV	collagen IV
Con A	Concanavalin A
COPD	chronic obstructive pulmonary disease
CSK	C-terminal SRC kinase
CYP	cytochrome P-450
Da	dalton
DAB	diaminobenzidine
dH_2O	distilled water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNA MTase	DNA-(cytosine-5) methyltransferase
dNTP	deoxynucleoside triphosphate
Dsh	dishevelled protein
E2F	early region factor 2
ECM	extracellular matrix
EDTA	ethylene diamino tetraacetic acid
EGF	epidermal growth factor
EGFr	epidermal growth factor receptor
ERK1	extracellular signal regulated kinase 1
ERK2	extracellular signal regulated kinase 2
FAK	focal adhesion kinase
FCS	foetal calf serum
Fen 1	flap endonuclease 1
Fn	fibronectin
g	grams
g	acceleration due to gravity
G0	gap 0
G1	gap 1
G2	gap 2
GADD45	growth and DNA damage protein
GSK-3	glycogen synthetase kinase
GTPase	guanosine-5'-triphosphatase

HCl	hydrochloric acid
H_2O_2	hydrogen peroxide
HPA	*Helix pomatia*
HRP	horseradish peroxidase
IAP	integrin-associated protein
Ig-CAM	immunoglobulin-cell adhesion molecules
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon-γ
IL	interleukin
ILK	integrin-linked kinase
IRS-1	insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
l	litres
LEF-1/Tcf	lymphoid enhancer binding factor/transcription factor
LFA-3	lysophosphatidic acid
Lam	laminin
M	mitosis
MAP	mitogen-activated protein
MAPK	mitogen activated protein kinase
MDM2	mouse double minute chromosome
MEK	MAPK-ERK kinase
MEKK-1	MEK kinase-1
mRNA	messenger ribonucleic acid
$MgCl_2$	magnesium chloride
MLCK	myosin light chain kinase
$MnCl_2$	manganese chloride
MPA	*Maclura pomifera*
µl	microlitres
ml	millilitres
mM	milli Molar
Na^+	sodium ion

NaOH	sodium hydroxide
NBT	nitrotetrazolium blue
NCAM	neural cell adhesion molecules
NFκB	nuclear factor κB
NGF	nerve growth factor
NLS	nuclear localisation signal
p21 ko	p21 knockout
PBS	phosphate buffered saline
PBST	phosphate buffered saline tween
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PECAM-1	platelet endothelial cell adhesion molecule-1
pH	$-\log_{10}[H^+]$
PI-3K	phosphatidylinositol-3-kinase
PMA	phorbol ester
Pol δ	DNA polymerase δ
Pol ε	DNA polymerase ε
R	restriction point
Rb	retinoblastoma
RER	rough endoplasmic reticulum
RGD	Arginine-Glycine-Aspartanine sequence
rpm	revolutions per minute
RPTPs	receptor protein tyrosine phosphatases
S	synthesis of DNA
SAPK	stress-activated protein kinases
SD	standard deviation
SDS	sodium dodecyl sulphate
SER	smooth endoplasmic reticulum
SP-A	surfactant protein A
SP-B	surfactant protein B
SP-D	surfactant protein D

STAT	signal transducer and activator of transcription
TBE	tris / boric acid / EDTA
TBS	tris buffered saline
TBST	tris buffered saline Tween-20
TCR	T-cell receptor
TE	tris EDTA solution
TGF-β	transforming growth factor-β
TNF	tumour necrosis factor
TPA	phorbol ester
TPO	thrombopoeitin
Tris	tris(hydroxymethyl) aminomethane
TSA	trichostatin A
UG	uteroglobin
UP1	urinary protein 1
UV	ultra violet (radiation)
V	volts
VCAM-1	vascular cell adhesion molecule-1
WAF	wild-type p53 activated factor
WGA	wheat germ antigen
WHO	World Health Organisation
wt	wildtype

Abstract

After lung injury epithelial cells are damaged may be shed thus denuding the basement membrane. The normal response to injury is usually an inflammatory reaction, deposition of new extracellular matrix that incorporates various growth factors, influx of inflammatory cells and regeneration of lost epithelial cells. Regeneration involves migration, proliferation and differentiation of stem cells to cover the exposed area. In the lung bronchiolar region Clara cells are thought to be the progenitor cells. Changes in cell cycle and behaviour of lung epithelial cells in several models of lung injury and *in vitro* primary culture were studied. The expression of the cell cycle control genes, p21, p53, PCNA and p27, in mouse models of three important human diseases mainly fibrosis, asthma and *Mycobacterium tuberculosis* infection were investigated. p21 expression by epithelial cells increased in fibrosis, decreased in asthma but did not change after TB infection. Clara cells were isolated from mice and cultured in serum free conditions for up to five days. They were characterised in terms of proliferation (BrdU, PCNA & mitosis), differentiation (lectins, integrins, cytokeratins & CC10), death (apoptosis and necrosis) and cell cycle regulation (p21, p53, EGFR & p27). The effects of cell matrix interactions on Clara cell proliferation, differentiation, cell cycle control and death were studied by varying the extracellular matrix composition and disruption of cell-integrin interactions in both wildtype and p21 knockout mice. Matrix changes did not influence significantly the proliferation, differentiation, death and cell cycle regulation in Clara cell cultures in both wildtype and p21 knockout mice, except when laminin was present. Significant differences in cytokeratin expression, cell cycle regulation and death pathways were observed in wildtype mice when compared to p21 knockouts. Some of the differences include lower expression of cytokeratin 8 and 19 in p21 ko mice, lower cytoplasmic but higher nuclear PCNA expression in p21 ko mice, a lower necrotic rate in p21 ko mice, lower cytoplasmic but higher nuclear p53 expression in p21 ko mice. By using a beta-1 blocking antibody the cell-integrin disruption was carried. Significant differences were observed upon beta-1 integrin blocking including an increase in cytokeratin 8 and 19 expression in p21 ko

mice, decrease in proliferation in both wt and p21 ko mice, an increase in cytoplasmic PCNA in both wt and p21 ko mice, an increase in apoptosis rate in both wt and p21 mice, increase in nuclear p21 in wt mice, an increase in nuclear p53 in both wt and p21 ko mice, and a decrease in cytoplasmic but an increase in nuclear p27. The two forms of p21 were studied and it was found that the cytoplasmic p21 is a C-terminal truncated protein with a weight of 17 kDa. The complex of p21 in both the cytoplasm and nucleus were studied. The nuclear p21 was found to form complexes with PCNA, cdk2, cdk4, cdk6, cyclin D3 and cyclin E, while the cytoplasmic p21 form complexes with cdk4 and cyclin D3.

In conclusion, the work presented in this thesis demonstrates that p21 is an important factor for the proliferation, differentiation and death of Clara cells. It also demonstrates that cell-matrix interactions form an important part in the regulation of Clara cells. Two forms of p21, a cytoplasmic and a nuclear protein are identified and their importance in the regulation and division of Clara cells is discussed.

Chapter 1 – Introduction

1.1 Biology of small airway epithelium.

The lung is the principal organ of gas exchange in the body and also carries out a number of non-ventilatory functions including humidification, thermal regulation, mucociliary clearance and elimination of volatile substances.

The lung is divided into three main zones, the conducting airways, respiratory bronchioles and alveolar airspaces. The conducting zone begins with the nasal cavity and mouth, and then opens through the larynx into the trachea. The trachea divides to form two bronchi that branch like a tree to form smaller passages, the bronchioles. The conducting airways serve as conductors of air from the external environment to the distal gaseous exchange area. The respiratory bronchioles, alveolar ducts and alveoli particulate in gas exchange area and are classified as the terminal respiratory unit, and are also called acinus (Figure 1.1).

The adult mammalian lung contains over 40 different cell types, of which at least 8 are found in the epithelial lining of the tracheobronchial airways. These epithelial cells include ciliated cells, basal cells, brush cells, mucous globlet cells, serous cells, Clara cells, type I, type II, neuroendocrine cells, and a variety of partially differentiated or intermediate cell types.

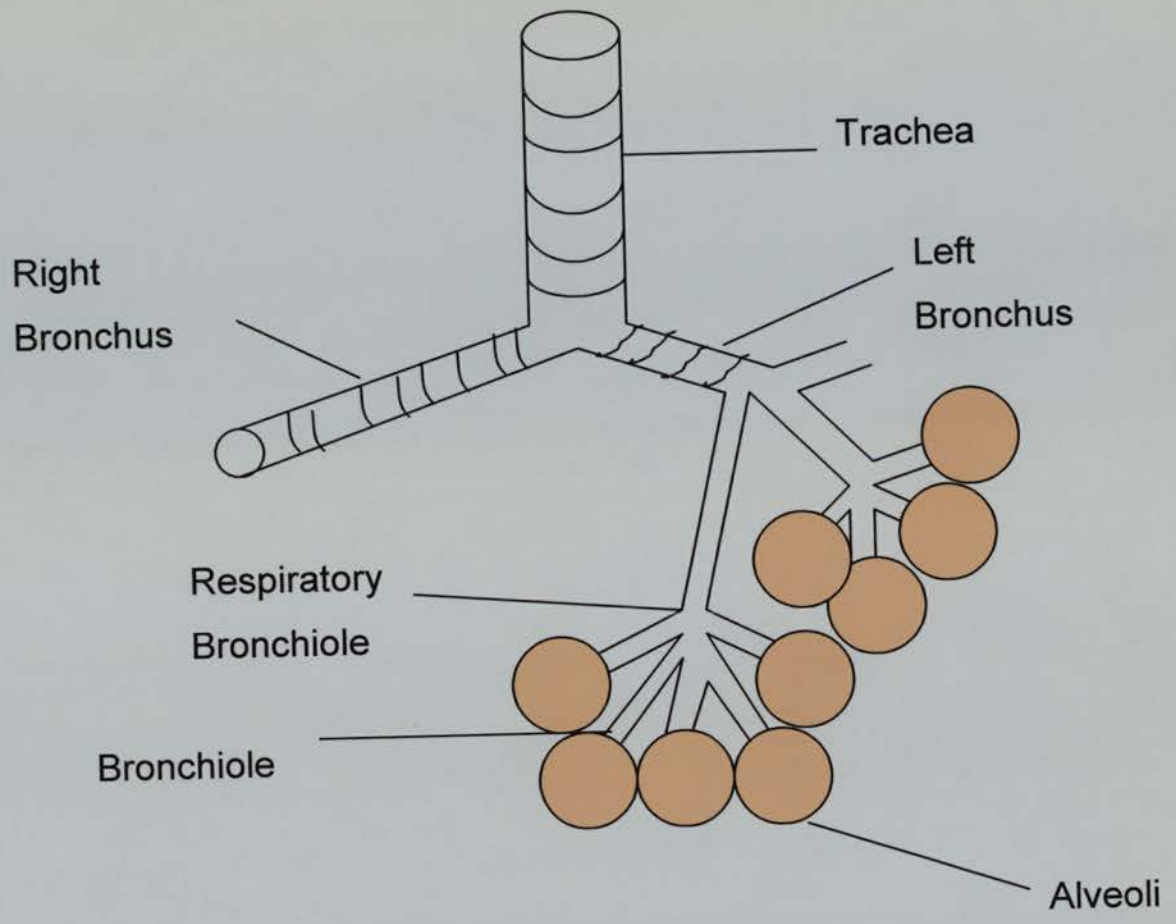


Figure 1.1 - Lung structure showing the trachea, left and right bronchiole, terminal bronchiole, bronchiole and alveoli regions.

1.2 The Respiratory Bronchioles.

The epithelium of the respiratory bronchioles is pseudostratified, having two or more rows of nuclei. It is composed of various cell types including ciliated, basal, mucous, serous, neuroendocrine and Clara cells. All epithelial cells in pseudostratified epithelium have contact with the basal lamina. (Figure 1.2)

1.2.1 Ciliated cells

Ciliated cells are terminally differentiated cells and although they are susceptible to injury from inhaled irritants are unable to divide. Therefore, they exfoliate easily and do not participate in the lung proliferative response (Ayers and Jeffery, 1988). These cells are approximately 20 μm high and have a columnar shape. They are attached to the adjacent cells by a junctional complex which consists of three elements, tight junctions (also known as zonula occludens) which form an impermeable barrier preventing macromolecules passing between cells, zonula adherens which link the actin filament network, and desmosomes which connect the intermediate filament network of adjacent cells such as basal cells (Frasca et al., 1968; Evans, 1973; Jeffery and Reid, 1975; Tátrai et al., 1994).

Ciliated cells have a basally or centrally placed nucleus. At ultrastructural level ciliated cells have a numerous mitochondria and basal bodies to which the cilia are attached. These cilia form a more or less continuous surface above large airways epithelium (Plopper and Dungworth, 1987). Their primary function is to sweep layers of mucus, together with trapped particulate material and dead cells. On average there are about 200 cilia per cell, although this number varies from a number of studies and their length is about 6 μm (Mariassy, 1992).

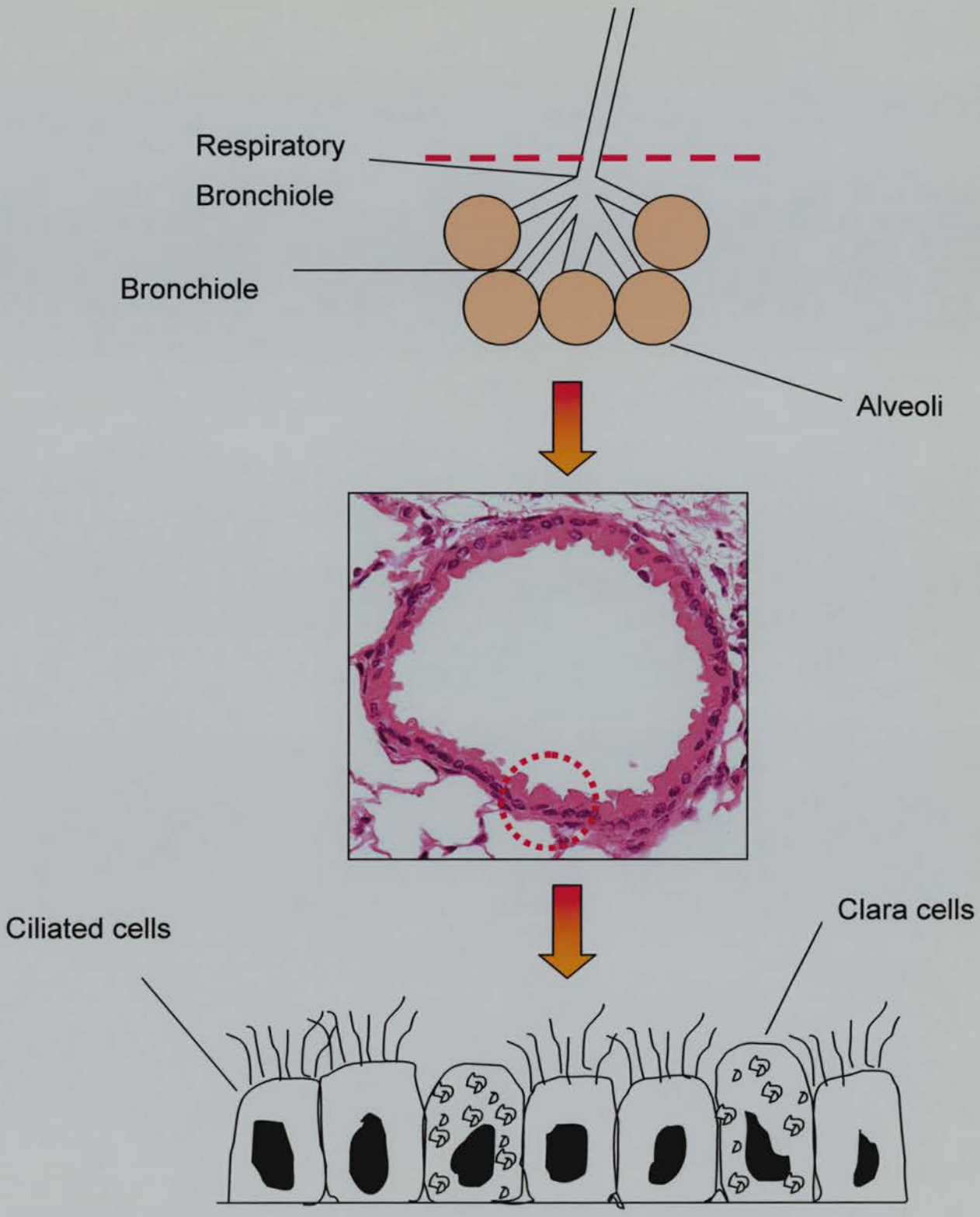


Figure 1.2 - Lung Bronchiolar region showing the cuboidal nature of the epithelium principally comprised of ciliated and interspersed with Clara cells.

1.2.2 Basal cells

Basal cells are numerous in the trachea and decrease in number from the trachea to the bronchioles. There are usually very few basal cells in the terminal bronchioles and none in the respiratory bronchioles. These cells are small (10 – 15 μm), with very small nuclear cytoplasmic ratio (Mariassy, 1992). They are round, triangular or flat in shape, with a dense round nucleus and a strongly stained cytoplasm. They are attached to the basal membrane with hemi-desmosomes and to the other epithelial cells through numerous desmosomes. They do not extend to the airway surface (Plopper et al., 1987).

The exact role of the basal cells is still unclear and under debate. In the earliest studies it was postulated that basal cells were the progenitor cells of the epithelium. Basal cells were thought to divide to form new basal cells as well as intermediate cells, capable of cell division and then, differentiate into mucous and ciliated cells. Evidence suggests that mucus-secreting cells were also capable of division and that basal cells do not seem to participate in the renewal of the respiratory epithelium. This debate is further complicated by the fact that there are substantial differences between species in the distribution and morphological characteristics of airway cell types (Evans et al., 1989; Inayama et al, 1988; Hicks et al., 1997; Erjefält et al., 1997; Boers et al., 1998; Nakajima et al., 1998).

1.2.3 Brush cells

Brush cells are non-ciliated epithelial cells and have been found in the conducting airways of a number of species. Brush cells are characterised by a densely packed of large, 2 to 3 μm long microvilli (Mariassy, 1992). The function of these cells has not been determined however a number of possible functions have been suggested, including chemoreception and monitoring the depth of the periciliary fluid. Brush cells are relatively rare, and always less that 1% of the cell population.

1.2.4 Mucous (Goblet cells)

In the trachea most cells are columnar, having round profiles when sectioned parallel to the basement membrane. Only rare cells have the classical goblet shape. The apical surface varies with stage of secretion. Junctional complexes make apical attachment of mucous cells while desmosomal attachments anchor the mucous cells to its neighbours or the lateral surfaces. Surface contact with the basal lamina are often lacking in longitudinal section due to the attenuated cytoplasm forming the stem (Mariassy, 1992).

Three dimensional reconstruction of a mucous goblet cell showed that the different planes of sections present a number of morphologic features associated with different stages of mucous cell secretion (Adler et al., 1982). Up to 60% of the mucous (goblet) cell profile is occupied by membrane-bound secretory granules.

The term mucous cells is commonly used for cells that contain relatively homogenous granules. The electron density of mucous cell granules may vary, since they lack the dense, osmiophilic, proteinaceous granule core or the surrounding halo.

Cells that contain such granules are referred to as sero-mucous cells. The organelles of the mucous cells include one or more Golgi bodies with associated vesicles and granular endoplasmic reticulum profiles between the secretory granules (Mariassy, 1992).

1.2.5 Serous cells

Serous cells are found in abundance in many glands in variety of animal species and humans (Basbaum et al., 1990). Depending on the source, the percentage of serous cell in a species varies from 27% (Jeffery and Reid, 1975) to 37% (Plopper et al., 1993) to 42% (Nikula et al., 1988). A cell type with generally serous cell

characteristics that contained disc- (0.13 μm) and rod- (0.4 μm) shaped granules were described in dogs (Frasca et al., 1968), humans (Plopper et al., 1980c; Plopper et al., 1980b; Plopper et al., 1980a) and cats (Jeffery and Reid, 1975; Ayers and Jeffery, 1988). These cells are referred to a specialised type cells but their function is not known. There are no quantitative data available on this cell (Mariassy, 1992).

1.2.6 Neuroendocrine cells.

Neuroendocrine cells have been referred to by numerous names, including helle zelle (clear cells), Feyrter's cells, Kultschitzky cells, APUD (amine precursor uptake and decarboxylation), endocrine-like cells, enterochromaffin-like cells, and small granule cells (Pearsall, 1989). Pulmonary neuroendocrine cells were shown to be progenitor cells and capable of self-renewal (Reynolds et al., 2000).

1.2.7 Clara cells (non-ciliated bronchiolar cells)

1.2.7.1 Ultrastructure

Clara cells, the nonciliated population in the epithelial lining of bronchioles, are one of the most heterogeneous and multifunctional cell types in the mammalian lung. The best-defined markers for Clara cell differentiation in adult mammals are the ultrastructural features (Table 1.1).

Clara cells are characterised as low cuboidal calls with minimal apical projections, bound to each other by junctional complexes on the luminal aspects of the basolateral membrane (Plopper et al., 1991; Massaro et al., 1994). The distribution of organelles within the cytoplasm shows little polarization and includes small amounts (<10%) of rough (RER) and smooth (SER) endoplasmic reticulum, mitochondria, and Golgi apparatus. The nucleus is approximately one-third of the total volume of the cells and is centrally placed. The apical portions of the cytoplasm generally contain a

small number of ovoid, electron-dense membrane-bound secretory granules. This structural composition has been defined for humans and in three species of macacque monkeys (Plopper et al., 1991).

The ultrastructure of the most of the mammalian species is slightly different from each other and is characterised by:

(i) extensive apical projections into the airways

(ii) a polarized organisation of organelles

In most species there is an abundance of apical smooth endoplasmic reticulum. In mouse, hamster, rat, guinea pig rabbit, pig, sheep and horse more than 40% of the cytoplasmic volume consists of smooth endoplasmic reticulum (Plopper et al., 1980b; Plopper et al., 1980c; Plopper et al., 1980a).

Component	Rabbit	Cat	Bonnet monkey
Cell	471.8 ± 51.8	496.1 ± 40.8	418.9 ± 36.1
Nucleus	83.0 ± 7.0	132.5 ± 45.6	119.9 ± 18.4
SER	213.4 ± 51.0	38.9 ± 24.4	15.6 ± 9.9
Glycogen	37.5 ± 13.1	222.9 ± 36.4	0
RER	25.8 ± 19.4	1.56 ± 3.38	38.9 ± 1.2
Mitochondria	75.4 ± 17.7	70.9 ± 34.9	42.0 ± 8.3
Granules	12.5 ± 6.7	0	6.3 ± 1.4
Golgi apparatus	5.7 ± 5.7	14.6 ± 13.1	24.1 ± 2.8

Table 1.1 - Comparison of volumes (μm^3) of cellular components in Clara cells adapted from (Plopper et al., 1991).

1.2.7.2 Clara cell functions

Clara cells have at least four roles in the normal lung function:

(a) contribute a secretion to the extracellular lining fluid

(b) progenitor cells for both themselves and for ciliated cells

(c) contain a variety of cytochrome p-450 monooxygenases that have an active role in metabolism of xenobiotics.

(d) regulate fluid balance in the distal conducting airways

1.2.7.2 a Secretion products.

Clara cells secrete a number of proteins including surfactants SP-A, B and D, Clara cell 10-kDa protein/uteroglobin, leukocyte protease inhibitors, β-galactoside binding lectin and a trypsin-like protease (Massaro et al., 1994; Peão et al., 1993; Cardoso et al., 1993). It was proposed that Clara cell section is both aprocrine and merocrine, the former prodominating (Peão et al., 1993).

Clara cells 10-kDa protein (CC10) is the predominant product of Clara cells and is distributed mainly in the bronchiole (Asabe et al., 1998; Sagal and Nieto, 1998; Singh and Katyal, 1997; Shijubo et al., 1999a; Xu et al., 1998). The protein has also been referred to as Clara cell 16-kDa protein according to results using electrospray/mass spectrometry. This protein was first identified in urine of patients with renal failure and purified later from lung lavage (Broeckaert and Bernard, 2000; Hermans et al., 1998a; Hermans et al., 1998b; Laing et al., 2000). The entire human CC16 gene has been sequenced and localised to chromosome 11, p12-q13, a region occupied by several genes involved in the regulation of allergy and inflammation (Broeckaert and Bernard, 2000). Human CC10 is identical to human urinary protein 1 (UP1) and human uteroglobin (UG) (Gutierrez and Nieto, 1998; Klug and Beato, 1996; Nord et al., 1998; Reynolds et al., 1999; Sagal and Nieto, 1998).

Changes in CC10 levels in sera and bronchoalveolar lavage (BAL) fluid have been reported in various lung diseases and in patients exposed to different toxins including

cigarette smoking (Asabe et al., 1998; Dodge et al., 1994; Shijubo et al., 1999a; Xu et al., 1998; Szabo et al., 1998; Laing et al., 2000; Shijubo et al., 1999b; Arsalane et al., 1999; Lesur et al., 1995; Johnston et al., 1998; Mango et al., 1998; Reynolds et al., 2000; Reynolds et al., 1999). Although the function of CC10 is unclear, it may play a role in regulation of inflammation.

Surfactant proteins and the mRNA for SP-B have been localised in the Clara cells (Phelps and Flores, 1991). The detection of SP-B in Clara cells is more consistent than that of SP-A. SP-A and SP-D are believed to be involved in host defence against micro-organisms by their lectin like activity (Kuan et al., 1992). The role of the surfactant proteins secreted by Clara cells is still unclear (Massaro et al., 1994; Margana and Boggaram, 1997).

The 29-kDa β-galactoside binding lectin may be the same as the 30-kDa Clara cell tryptase, but their function and physiological role is still not clear. Clara cell tryptase has been shown to cleave haemagglutinin and activate infectivity of influenza A virus (Sakai et al., 1993; Tashiro et al., 1996; Kohri et al., 1996; Kido et al., 1997; Wasano and Yamamoto, 1989)

The leukocyte protease inhibitor has been immuno-histochemically localised to Clara cells. This protein inhibits leukocyte proteases, especially elastase (De Water et al., 1986; Willems et al., 1989).

1.2.7.2 b Progenitor cells.

Clara cells are self replicative but also terminally differentiate into ciliated cells in the bronchioles (Plopper et al., 1991; Plopper et al., 1992b; Ji et al., 1995; Ayers and Jeffery, 1988). Clara cells isolated from the lungs of rabbit were shown to be able to re-populate denuded tracheas (Hook et al., 1987). Clara cells were shown not to be necessary for pulmonary neuroendocrine cell hyperplasia (Reynolds et al., 2000).

1.2.7.2 c Metabolism of xenobiotics.

Among the other epithelial cells found in the lung epithelial bronchiolar region, Clara cells are distinguished for their ability to metabolise xenobiotics via the cytochrome P-450 (CYP) monooxygenase system and flavin-containing monooxygenases (Overby et al., 1992; Buckpitt et al., 1992; Plopper et al., 1993; Gebremichael et al., 1995; Buckpitt et al., 1995; Lakritz et al., 1996; Watt et al., 1998). Bronchiolar Clara cells are considered to be one of the principal targets in the mammalian lung pulmonary toxicants. These include a wide variety of compounds including furans, chlorinated hydrocarbons, and aromatic hydrocarbons. Many compounds injure Clara cells in most of the species but Clara cells in different species do not have the same level of sensitivity to any one compound (Voigt et al., 1989; Baron and Voigt, 1990; Buckpitt et al., 1992; Plopper et al., 1992a; Plopper et al., 1992c; Buckpitt et al., 1995).

Changes in Clara cells are manifested as early as 1 hour after exposure and involve clumping and margination of nuclear chromatin, mitochondrial swelling, dilation of endoplasmic reticulum membrane and disruption of cell junctions. Within 24 hours of exposure the injury there is cellular enlargement and formation of large numbers of membrane-bound vacuoles (Chichester et al., 1991; Plopper et al., 1991; Plopper et al., 1992a; Plopper et al., 1992c; Buckpitt et al., 1992; Massaro et al., 1994; Lakritz et al., 1996; Van Winkle et al., 1996a; Buckpitt et al., 1995).

There are a large number of studies showing the ability of Clara cells to metabolise a number of xenobiotics including naphthalene and its metabolites (Kanekal et al., 1991; Buckpitt et al., 1995; Kanekal et al., 1990; Plopper et al., 1992a; Buckpitt et al., 1992; Plopper et al., 1992c; Van Winkle et al., 1995; Lakritz et al., 1996; Van Winkle LS et al., 1996; Sauer et al., 1997; Fanucchi et al., 1997; Van Winkle et al., 1997; Zheng et al., 1997; Paige et al., 1997), ozone (Castleman et al., 1980; Nikula et al., 1988; Dodge et al., 1994; Royce and Plopper, 1997; Watt et al., 1998; Mango et al., 1998; Arsalane et al., 1999), styrene and its compounds (Male et al., 1985; Cruzan et al., 1997), benzo(α)pyrene and its derivative compounds (Bend et al.,

1981; Jones et al., 1982b; Sivarajah et al., 1983; Horton et al., 1985; Stowers and Anderson, 1985; Kontir et al., 1986; Overby et al., 1992; Boutin et al., 1998; Wu et al., 1998b; Jyonouchi et al., 1999), cigarette smoke (Ji et al., 1994; Gebremichael et al., 1995; Ji et al., 1998), coumarin (Born et al., 1998; Born et al., 1999), methylene chloride (Foster et al., 1994; Green, 1997), trichloroethylene (Green et al., 1997; Giovanetti et al., 1998), amines (Masek and Richards, 1990), and nitrogen dioxide (Evans et al., 1986). Dially sulfone, a derivative of garlic, was found to protect against Clara cell injury caused by 1,1-dichloroethylene by conjugating a glutathione with its reactive metabolites (Forkert et al., 1996; Forkert, 1997; Forkert, 1998; Forkert et al., 1999).

1.2.7.2 d Regulation of fluid balances.

Due to the fact that bronchiolar epithelium has lateral projections, it has been suggested that the airways epithelium of distal conducting airways is involved in re-absorption and clearance of airway-lining fluid (Plopper and Dungworth, 1987; Van Scott et al., 1987). It was shown that there is a net movement of sodium ions (Na^+) from the mucosal to the serosal side in cultured monolayers of rabbit Clara cells. This movement is amiloride-sensitive and occurs under short- or open-circuit conditions, thus most likely Clara cells are involved in fluid re-absorption. Chloride ions (Cl^-) secretion was observed to be induced by amiloride, but no net Cl^- movement was observed under basal conditions (Van Scott et al., 1989). Cl^- channels that can be stimulated by c-AMP-activating agents and extracellular ATP were described in Clara cells. These channels share many bio-physiological properties with the Cystic Fibrosis Transmembrane Regulator (CFTR)-related Cl^- channel (Chinet et al., 1997).

1.3 The Alveoli

The alveoli are the principal site of gas exchange in the lung. The alveolar epithelium is composed of two cell types Type I and Type II cells (Evans et al., 1975; Jones et al., 1982bb; Jones et al., 1982a; Sivarajah et al., 1983; Devereux, 1984; Horton et al., 1985; Devereux et al., 1985; Dobbs et al., 1985; Domin et al., 1986; Dobbs et al., 1986; Marshall et al., 1988; Honda et al., 1989; Belinksy et al., 1992; Belinksy et al., 1995; Harrison et al., 1995; Corti et al., 1996; Strayer et al., 1998; Williams, 1999) (Figure 1.3).

1.3.1 Type I cells

Type I cells are squamous cells which cover 90% of the alveolar surface. The thin and attenuated cytoplasm of type I cells facilitates gas exchange by minimising the diffusion distance from alveolar to the blood.

1.3.2 Type II cells

Type II cells are cuboidal cells and occupy 10% of the alveolar surface. The primary functions of type II cell are:

(a) synthesis and secretion of surface-active material

(b) maintenance of the alveolar epithelium by their ability to proliferate and to differentiate into type I cells

(c) trans-epithelial transport of sodium from the apical to the basolateral surface to minimize alveolar fluid.

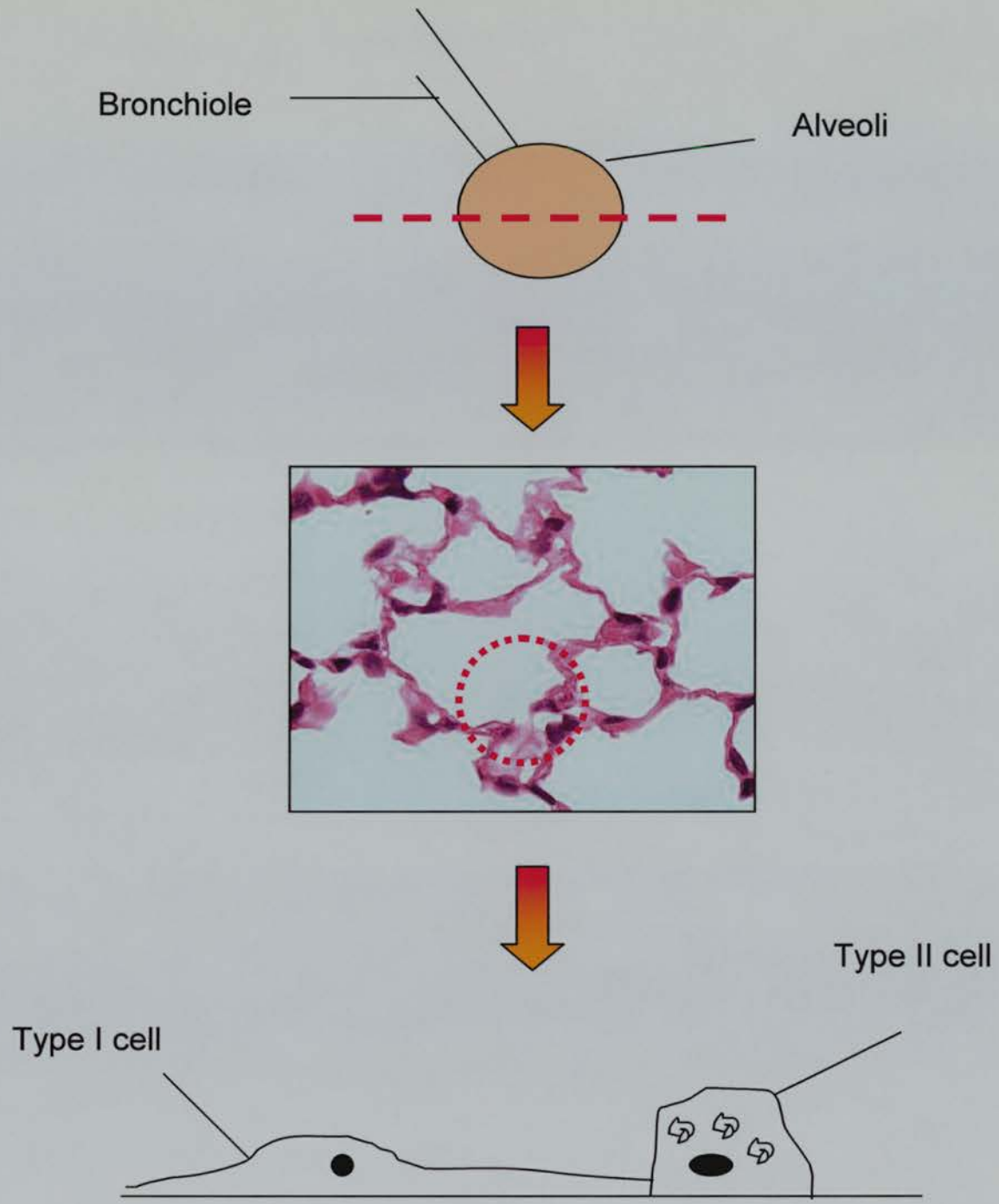


Figure 1.3 - The lung alveolar epithelium region showing the squamous nature of the epithelium comprised of the extremely thin Type I cells and the cuboidal Type II cells.

1.4 Epithelial cell repair.

Airways are exposed to a number of inhaled toxins, pathogens, allergens, reactive gases, aerosols and irritant particles. After a lung insult, some of the epithelial cells are injured or damaged. These cells either detach or die thus disruption of cell-cell and cell-matrix interaction and also exposing the basement membrane. The normal response to the injury is a mass transportation of factors including extra cellular matrix (ECM), various growth factors and inflammatory cells. The epithelial cells in the margin of the damage flatten and move to eventually cover the wound (Rennard, 1999; Erjefält et al., 1995; Persson, 1996; Persson et al., 1996; Erjefält et al., 1996; Erjefält et al., 1997; Erjefält and Persson, 1997). If this repair does not occur this insult can result in a disease (Figure 1.4).

So far there are little data showing which cell types are involved and by what mechanism this repair takes place. There are two main hypothesis how the repair might take place. The first model postulates that basal cells act as the principal stem cell in the conducting airways, giving rise to the various secretory cells and ciliated cells. This model is based on the cell turnover studies of normal respiratory epithelium (Nettesheim et al., 1990). The other model postulates that the secretory cells are the progenitor cells from which most of the other cells develop. The evidence on this second model is based from the regeneration of chemically or physically injured epithelium (Nettesheim et al., 1990).

The most common basally situated cell, the 'basal' cell was described and proposed as a progenitor cell (Blenkinsopp, 1967). In peripheral bronchioles where basal cells are absent, the Clara cell is the progenitor cell (Evans, 1973; Evans et al., 1978; Evans et al., 1986). Clara cells may divide in response to epithelial irritation and subsequently differentiate to form mature secretory and ciliated cells (Hook et al., 1987; Cardoso et al., 1993). In the alveolus, the type II cell is the progenitor cell from which the type I cell differentiate (Castleman et al., 1980; Evans, 1973; Evans et al., 1975).

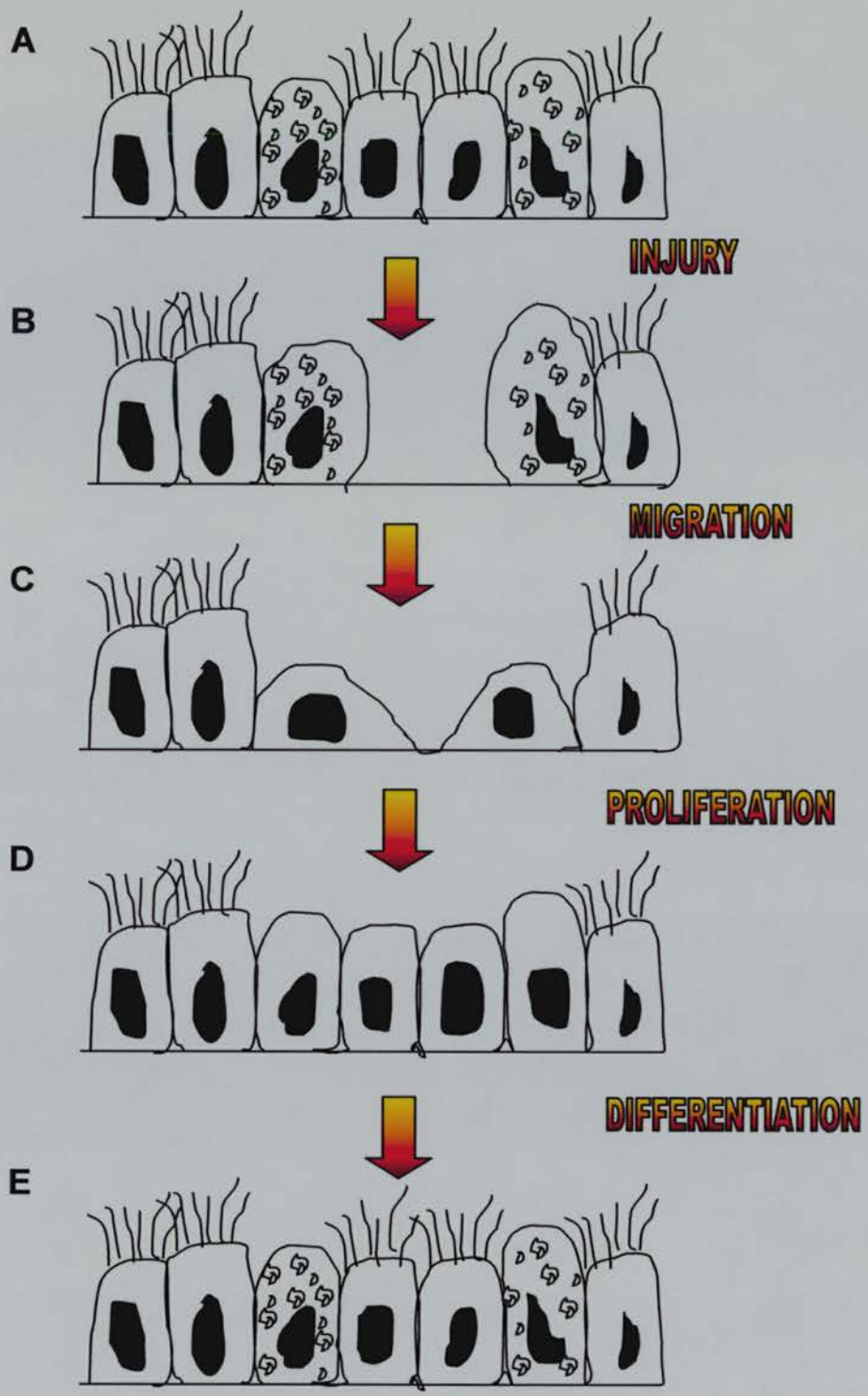


Figure 1.4 - Stages of injury and repair in lung bronchiolar region (A) Normal epithelium (B) After an injury portions of basement membrane is denuded (C) provisional matrix is deposited and neighbouring cells migrate to fill up the gaps in the epithelium (D) Progenitor cells proliferate and (E) cells differentiate in order to restore the epithelium.

1.5 Epithelial cell interactions

Cell interactions are an important factor for the maintenance of both three-dimensional structure and normal function in tissues. The biochemical entities mediating cell adhesion are multiprotein complexes comprising three broad classes of macromolecules: the adhesion receptors, the extracellular matrix molecules and the adhesion plaque proteins (Gumbiner, 1996).

The extracellular matrix has been traditionally viewed as either forming the structurally stable support for cells and tissues or as being responsible for the basement membrane formation (Puchelle and Zahm, 1996). It is known that extracellular matrix-cell interactions regulate the morphology and cell functions through a family of specific cell surface receptors – the integrins (Dunsmore and Rannels, 1996; Chintala and Rao, 1996; Kettritz et al., 1999; Vitale et al., 1999; Boudreau and Jones, 1999; Streuli, 1999; Geiger et al., 2001).

Cell adhesion receptors are typically transmembrane glycoproteins that mediate binding to extracellular matrix (ECM) molecules or to counter-receptors on other cells. These molecules determine the specificity of cell-cell and cell-ECM interactions. The ECM proteins are usually fibrillar in nature and provide a complex structural and functional nature that can interact simultaneously with multiple cell surface receptors. The peripheral membrane proteins provide structural and functional linkages between adhesion receptors and the actin microfilaments, microtubules, and intermediate filaments of the cytoskeleton (Howe et al., 1998; Aplin et al., 1998).

1.5.1 Extracellular Matrix (ECM)

Extracellular matrix (ECM) is a general term that encompasses components of the basement membrane and interstitial connective tissue. The extracellular matrix contains signals that control cell shape, migration, proliferation, differentiation, morphogenesis and survival (Lukashev and Werb, 1998; Boudreau and Jones, 1999; Streuli, 1999). The basement membrane comprises collagen IV, laminins, entactin, fibronectin and proteoglycans while the interstitial connective tissue is made of fibrillar collagens, elastic fibers and proteoglycans (Sannes and Wang, 1997; Dunsmore and Rannels, 1996; Lukashev and Werb, 1998; Coraux et al., 1998; Klinowsks et al., 1999; Ploplis et al., 2000; Arroyo et al., 2000; Costa et al., 2001). Interstitial connective tissue is important during development and wound repair (Lukashev and Werb, 1998; Coraux et al., 1998; Klinowsks et al., 1999; Ploplis et al., 2000; Arroyo et al., 2000; Costa et al., 2001).

ECMs act in concert with other signalling pathways, such as those initiated by growth factors, to regulate cell behaviour (Damsky and Werb, 1992; Boudreau and Jones, 1999; Geiger et al., 2001). Cells use a series of receptors for ECM including integrins (Hynes, 1992; Lasky, 1997; van der Flier and Sonnenberg, 2001), cell surface proteoglycans (Xu et al., 1996; Page, 1997), and cell-surface-expressed tyrosine kinase receptors with direct affinity for ECM (Schlessinger and Ullrich, 1992; Vogel et al., 1997). The components of ECM include insoluble ECM proteins such as collagens, laminins, fibronectin and proteoglycans, matricellular ECM proteins that modulate cell-matrix interactions and other cellular responses such as cell proliferation and ECM-associated proteins such as growth factors (Roskelley et al., 1995; Lukashev and Werb, 1998; Damsky and Werb, 1992; Dunsmore and Rannels, 1996; Streuli, 1999; Boudreau and Jones, 1999; Geiger et al., 2001; Talpale and Keski-Oja, 1997).

During tissue injury, the composition of ECM and its cellular recognition sites are altered in a number of significant ways. Increased vascular permeability results in

recruitment of plasma-derived proteins including fibronectin, vitronectin and fibrinogen into the ECM, whereas cells in the injury site are induced to release or synthesise new components including thrombospondins, tenascins and alternatively spliced fibronectins which regulate tissue repair (Chintala and Rao, 1996; Kettritz et al., 1999; Vitale et al., 1999; Krüger-Krasagakes et al., 1999; Ebihara et al., 2000). Furthermore, tissue injury may result in alterations in existing ECM proteins within tissues or in recruited ECM that reveal cryptic biologically active sites (matricryptic) that provide important signals within the injury sites (Davis et al., 2000; Montgomery et al., 1994).

Among the extracellular matrix proteins, fibronectin matrices appear critical to tissue repair. Following tissue injury, the expression of fibronectin increase dramatically (Reynolds et al., 1999; Sottile et al., 2000; Miyata et al., 2000; Danen and Yamada, 2001). Much of the fibronectin detected in injured lungs is of plasma origin, but it is also locally synthesised by macrophages, fibroblasts, endothelial cells, and surface epithelial cells (Lukashev and Werb, 1998; Dunsmore and Rannels, 1996; Chintala and Rao, 1996; Kettritz et al., 1999; Streuli, 1999; Ebihara et al., 2000; Davis et al., 2000). Fibronectin may serve multiple functions in tissue repair, acting as a chemo-attractant and adhesive substrate for mesenchymal and epithelial cells, which migrate into damaged tissue (Limper and Roman, 1992). In addition to fibronectin, two components of the basement membrane, laminin and type IV collagen, can stimulate bronchiole epithelial cell migration (Crystal et al., 1978; Sannes et al., 1998; Sannes et al., 1998; Crystal et al., 1978; Vogel et al., 1997).

1.5.2 Integrins

The integrins are a family of cell-surface glycoproteins that act as receptors for ECM proteins or for membrane-bound counter-receptors on other cells (Ingber, 1991; Hynes, 1992; Schwartz, 1997; van der Flier and Sonnenberg, 2001). Integrins mediating cell-ECM adhesion sites are complex specialised structures termed focal contacts or focal adhesions (Turner, 2000). Each integrin is a heterodimer that

contains an α and a β subunit, each subunit having a large extracellular domain, a single membrane-spanning region and in most cases (other than β_4), a short cytoplasmic domain (Hynes, 1992; van der Flier and Sonnenberg, 2001).

The integrin receptor family of vertebrates include at least 16 distinct α subunits and 8 or more β subunits which can associate to form more than 20 distinct integrins (Hynes, 1992; Hynes, 2000). The α/β pairings specify the ligand-binding abilities of the integrins heterodimers. Although the ligands for integrins are often large ECM proteins such as collagen, laminin, vitronectin, or fibronectin, some integrins recognise rather short peptide sequences within the larger proteins, for example, the RGD (Arg-Gly-Asp) sequence found in the fibronectin and vitronectin. In other cases, however, integrin-ligand recognition depends on the overall conformation of the ligand protein. For example, some integrins interact with members of other adhesion receptor families, including Ig-Cams and cadherins, in a manner that does not involve RGD motifs (Kumar, 1998). Some integrins such as $\alpha_5\beta_1$ bind to a single ECM protein in this case fibronectin (Danen and Yamada, 2001), while other integrins such as $\alpha_v\beta_3$ has been reported to bind to vitronectin, fibrinogen, von Willebrand factor, thrombospondin, fibronectin, osteoporotin and collagen (Hynes, 1992; Fornaro and Languino, 1997; van der Flier and Sonnenberg, 2001) (Table 1.2). Cells often display multiple integrins capable of interacting with a particular ECM protein, thus integrin expression is often apparently redundant, at least in terms of simple cell adhesion. Some integrin subunits undergo alternative splicing of their cytoplasmic domain regions in a tissue-type specific and developmentally regulated manner, which suggests that there are discrete intracellular functions for individual integrins (Schwartz and Assoian, 2001; O'Toole, 1997; Clark and Brugge, 1995).

Subunits		Ligands and counter-receptors
β_1	α_1	Collagens, laminin, fibronectin
	α_2	Collagens, laminin
	α_3	Collagens, laminin, fibronectin
	α_4	Fibronectin, VCAM-1
	α_5	Fibronectin
	α_6	Laminin
	α_7	Laminin
	α_8	Fibronectin, tenescin
	α_9	Vitronectin, fibronectin
	α_{10}	Collagen
	α_{11}	Collagen
β_2	α_L	ICAM-1, ICAM-2
	α_M	ICAM-1, fibrinogen, factor X
	α_X	Fibrinogen
β_3	α_{IIb}	Fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin
	α_v	Vitronectin, fibrinogen, von Willebrand factor, thrombospondin, fibronectin, osteoporotin, collagen
β_4	α_6	Laminin
β_5	α_v	Vitronectin
β_6	α_v	Fibronectin
β_7	α_4	Fibronectin, VCAM-1
	α_E	?
β_8	α_v	?

Table 1.2 - The integrin receptor family and putative ligands. (Hynes, 1992; Kumar, 1998; Damsky and Werb, 1992; Fornaro and Languino, 1997; Sheppard, 2000; van der Flier and Sonnenberg, 2001)

The integrin cytoplasmic domains determine the interactions between the extracellular environment, intracellular structure and cascades signalling. Both the α and β subunit cytoplasmic domains make important contributions to various aspects of overall integrin function including cytoskeletal organisation, cell motility, signal transduction, and modulation of integrin affinity for ligands (Ingber, 1991; Hynes, 1992; Schwartz, 1997; Frisch and Ruoslahti, 1997; Lasky, 1997; Shyy and Chien, 1997; van der Flier and Sonnenberg, 2001).

The α subunit cytoplasmic domain inhibits certain functions of the β cytoplasmic domain (eg, the focal contact recruitment) but the binding of a ligand to the integrin relieves this inhibition, possibly by allowing the subunits to swing apart like a hinge (Lasky, 1997; Aplin et al., 1998). The β cytoplasmic domain is also important in signal transduction, particularly integrin activation of FAK, whereas the truncation/mutation of the α cytoplasmic domain has little effect on this process (Mould et al., 1996; Vasioukhin et al., 2000)

Several cytoplasmic proteins including talin, α-actinin, and possibly focal adhesion kinase (FAK) bind directly to the β_1 cytoplasmic domain and contribute to integrin-cytoskeletal interactions and bidirectional transmembrane signalling (Ingber, 1991; Clark and Brugge, 1995; Maniotis et al., 1997; Aplin et al., 1998; Schwartz and Assoian, 2001; Schlaepfer and Hunter, 1998; Laflamme et al., 1997; Dedhar and Hannigan, 1996). Table 1.3 summarises the various intracellular proteins that interact with integrin subunit cytoplasmic domains.

Proteins		Integrin subunit(s)
Cytoskeletal Proteins	α-actinin	β_1, β_2, β_3
	Talin	β_1, β_2, β_3
	Filamin	β_2
	Tensin, vinculin, paxillin, actin	β_1
	F-actin	α_2
Regulatory and signal transducing proteins	Endonexin	β_3
	Calreticulin	α_2, α_3, α_4, α_v, α_6
	IAP	α_v
	CD9, CD63	$\alpha_3\beta_1$
	FAK	β_1, β_2, β_3
	ILK	β_1, β_2, β_3
	IRS-1	$\alpha_v\beta_3$
	p-190	$\alpha_v\beta_3$
Other signalling molecules	C-CSK, P13K, Rho, Ras, Grb2, MEKK, MEK, ERK1, ERK2, CAS, Src family kinases	?

Table 1.3 Intracellular proteins suggested to interact with integrin subunit cytoplasmic domains. (Hemler, 1998; Dedhar and Hannigan, 1996)

Integrins activate a number of a large array of signalling intermediates. Their effects include activation of Rho-family GTP-ases leading to changes in cytoskeletal organisation, activation of mitogen-activated protein (MAP) kinase pathways, and activation of an array of proteins and lipid kinases. These signalling pathways allow integrins to influence cell-cycle progression, cell survival and gene expression in addition to their effect on cell adhesion and morphology (Hynes, 2000; Clark and Brugge, 1995; Hemler, 1998; Cary et al., 1999; Howe et al., 1998; Giancotti, 1997; Giancotti and Ruoslahti, 1999; Pu and Streuli, 2002; Schwartz and Assoian, 2001; O'Toole, 1997; Schlaepfer and Hunter, 1998; Carmeliet, 2002). In fact, most cells will not proliferate or survive unless they are adhering to a substrate – so called anchorage dependence (Schulze et al., 1996; Day et al., 1997; Schwartz, 1997; Orend et al., 1998).

Various biological responses are affected by integrins, including cytoskeletal organisation and cell spreading (Schwartz, 1997; Maniotis et al., 1997), cell motility (Ingber, 1991; Hynes, 1992; Shyy and Chien, 1997; Frisch and Ruoslahti, 1997; Maniotis et al., 1997), cell survival (Shyy and Chien, 1997) and cell proliferation (Vivinus-Nebot et al., 1999; Schwartz and Assoian, 2001). A simplified pathway showing the signalling mediated adhesion receptors and signalling intermediates is shown in Figure 1.5.

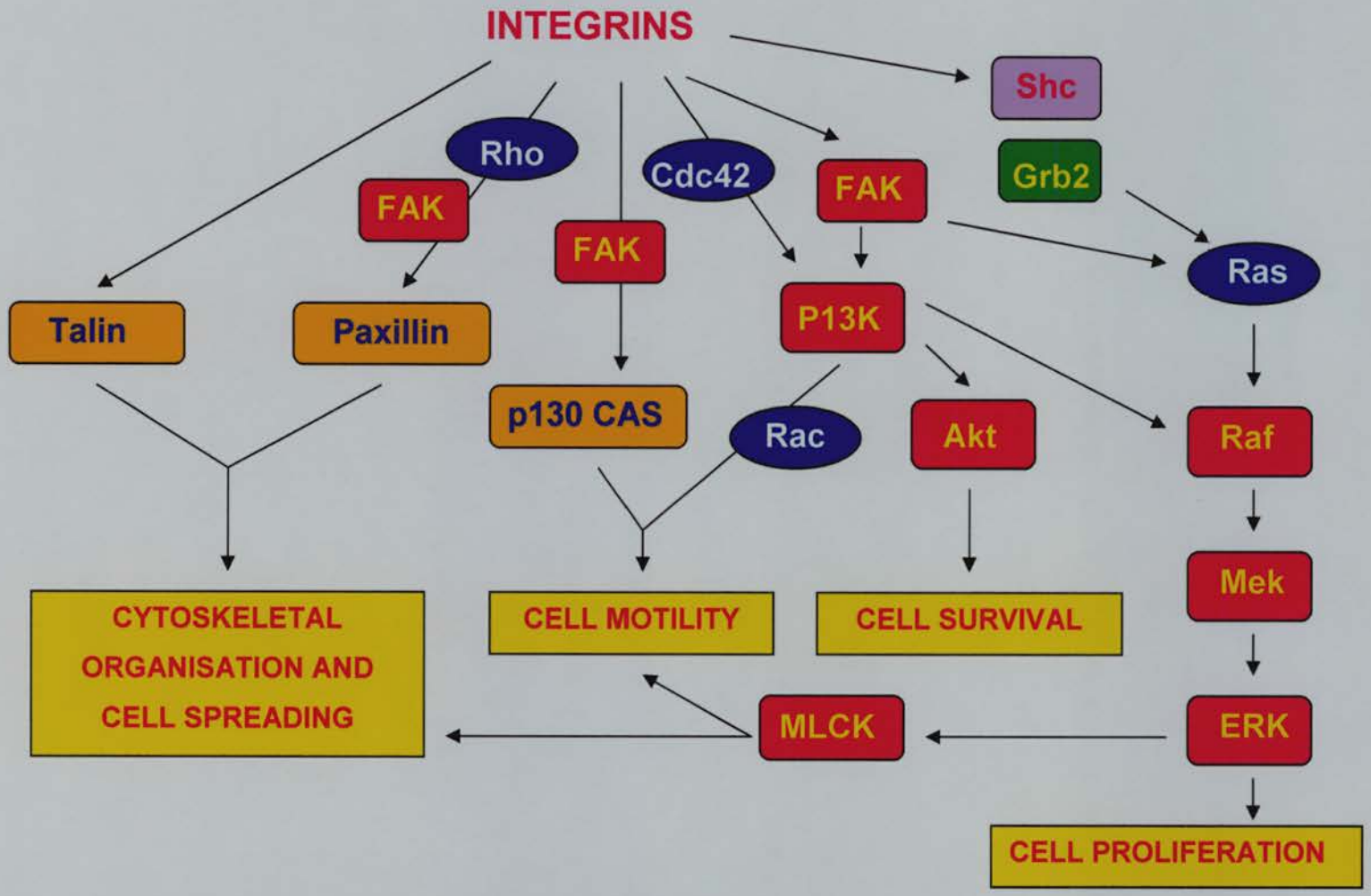


Figure 1.5 - Summary of the integrin signalling intermediates, including small GTPases (blue), protein kinases (red) cytoskeletal proteins (orange), and others. Integrins can affect many biological processes (yellow). Adapted from (Hynes, 2000).

1.5.3 Cadherins

Cadherins comprise a family of transmembrane proteins that share an extracellular domain consisting of multiple repeats of a cadherin-specific motif (Suzuki, 1996). The "classical" subfamily is calcium dependent homotypic cell-cell adhesion molecules. Several members which fall in this subfamily have been characterised including N-, P-, R-, B- and E-cadherin as well as approximately 10 others (Takeichi, 1995). These molecules are present in specialised sites of cell-to-cell adhesion (termed adherence junction), whereby they can establish linkages with the actin-containing cytoskeleton known as "adhesion zippers" (Aplin et al., 1998). The classical cadherins play a key role in developmental processes (Takeichi, 1995; Reithamacher et al., 1995).

Another important subfamily of cadherins involved in the adhesion is represented by the desmogleins and desmocollins, a group of desmosome-associated cadherins that form intracellular linkages to intermediate filaments rather than actin filaments (Cowin and Burke, 1996).

The cytoplasmic domains of cadherins interact strongly with a group of intracellular proteins known as catenins. It was shown that catenins are essential for cadherin function. Truncation of the cadherin cytoplasmic domain to delete catenin binding sites lead to a loss of cadherin-mediated adhesion (Takeichi, 1995; Gumbiner, 1996). Due to the fact that different classic cadherins have considerable homology among their cytoplasmic domains, they compete for the same catenins (Kinter, 1992). Three forms of catenin proteins have been described, α-, β- and γ-catenin. The structure of α-catenin shows substantial homology to the protein vinculin, which binds α-actinin and talin and is critical for cytoskeletal assembly at integrin-mediated focal adhesion sites (Aberle et al., 1996). β-catenin binds directly to the cadherin cytoplasmic domain, subsequently, α-catenin binds to β-catenin and links the complex to the actin cytoskeleton by direct interaction with actin and by binding α-actinin (Cowin

and Burke, 1996). β-catenin does not only interacts with cadherins but also with components of the wingless/Wnt signalling pathway and thus can stimulate transcription of target gene (Moon et al., 1997; Peifer, 1996; Bremnes et al., 2002) (Figure 1.6).

1.5.4 Immunoglobulin-Cell adhesion molecules (Ig-CAMs)

Immunoglobulin superfamily cell adhesion molecules (Ig-CAMs) are defined by the presence of one or more copies of the Ig fold, a compact structure with two cysteine residues separated by 55 to 75 amino acids arranged as two anti-parallel β sheets (Vaughn and Bjorkman, 1996). In many cases, Ig-CAMs contain one or more copies of a fibronectin type III repeat domain (Aplin et al., 1998).

Some examples of the Ig-CAMs superfamily include: neural cell adhesion molecules (NCAM) (Baldwin et al., 1996; Tessier-Lavigne and Goodman, 1996; Edelman and Crossin, 1991), EpH (Tessier-Lavigne and Goodman, 1996), CD2, CD4, CD8 (Dustin and Springer, 1991; Springer, 1995), intercellular adhesion molecule 1 & 2, (ICAM-1, ICAM-2) (Rosales and Juliano, 1995; Dustin and Springer, 1991; Springer, 1995), T-cell receptor (TCR) (Dustin and Springer, 1991; Springer, 1995; Rosales and Juliano, 1995), lysophosphatidic acid (LFA-3) (Aplin et al., 1998), vascular cell adhesion molecule-1 (VCAM-1) (Aplin et al., 1998), platelet endothelial cell adhesion molecule-1 (PECAM-1) (DeLessier et al., 1994), receptor protein tyrosine phosphatases (RPTPs) and others.

Ig-CAMs play multiple roles in the developing embryo and in the adult organisms. They are important in tissue organisation and cellular trafficking in the immune system (Aplin et al., 1998).

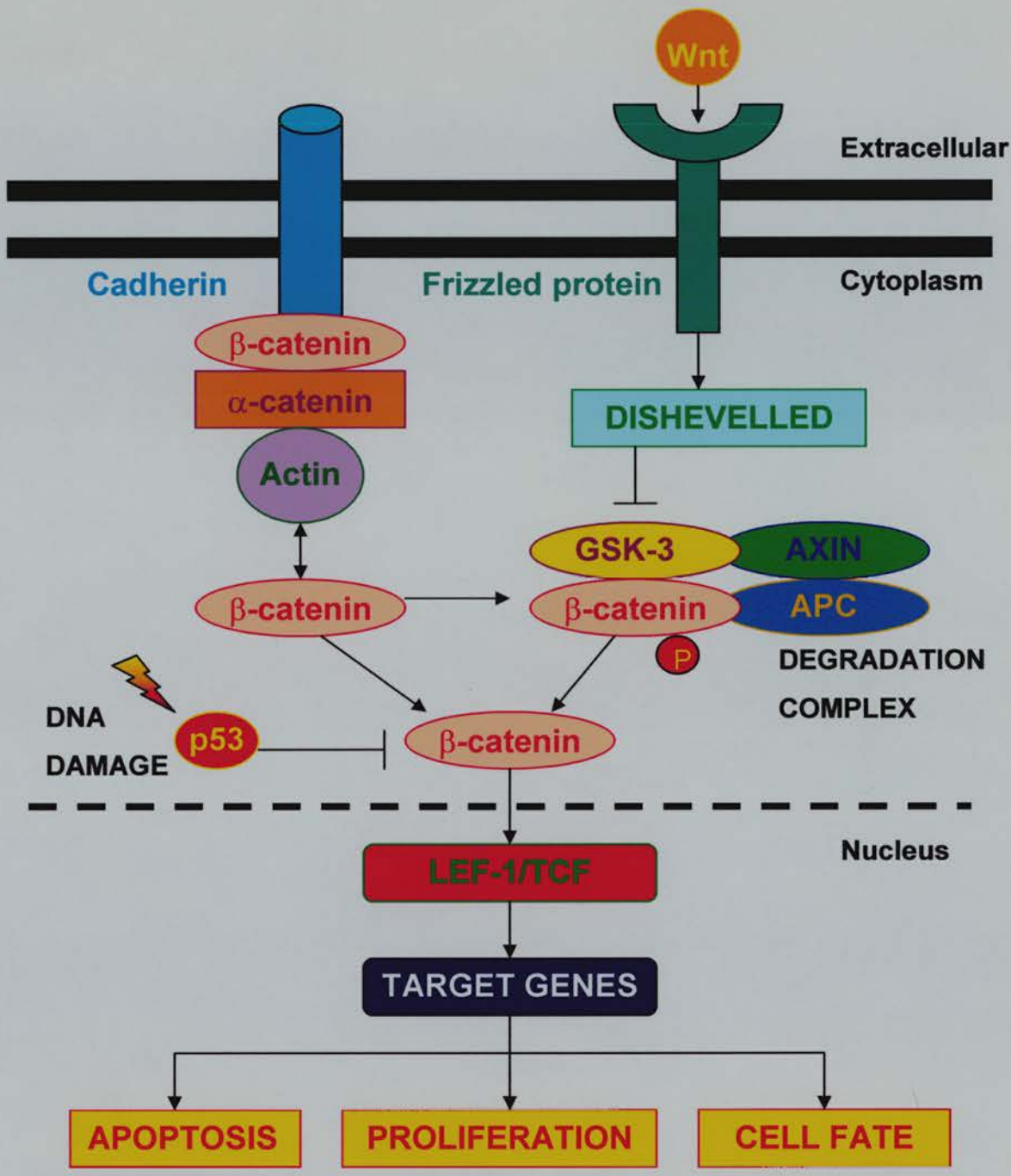


Figure 1.6 - Schematic presentation of the Wnt/β-catenin pathway. In absence of a mitotic signal, β-catenin is sequestered in a complex with adenomatous polyposis coli (APC), glycogen synthetase kinase (GSK-3) and scaffolding protein axin and is phosphorylated by GSK-3, enabling its degradation. An activation of Wnt pathway leads to activation of dishevelled (Dsh) protein which downregulates the complex so that it no longer phosphorylates β-catenin. The increased level of free β-catenin translocates to the nucleus, bind to transcription factors (LEF-1/Tcf), and stimulate transcription of target gene. Free β-catenin can be inhibited by p53. (Aplin et al., 1998; Bremnes et al., 2002; Moon et al., 2002).

1.5.5 Selectins

Selectins are a small family of lectin-like adhesion receptors composed of three members, L-, E- and P- selectin (Lasky, 1995; Rosen and Bertozzi, 1994; Tedder et al., 1995). The structure of a selectin includes an amino-terminal domain that is homologous to calcium-dependent animal lectins, followed by an epidermal growth factor (EGF)-type domain, two to nine complement regulatory protein repeats, a transmembrane helical segment, and a short cytoplasmic tail (Aplin et al., 1998). Selectins mediate heterotypic cell-cell interactions through calcium-dependent recognition of sialyated glycans. The physiological role of selectins concerns leukocyte adherence to endothelial cells and platelets during inflammatory processes (Rosales and Juliano, 1995; Springer, 1995).

P-selectin is present in latent form in endothelial cells and platelets and it is rapidly translocated from secretory granules to the cell surface upon activation by thrombin or other agonists (Rosen and Bertozzi, 1994; Lasky, 1995; Tedder et al., 1995). P-selectin was found to be upregulated in bleomycin induced injury in rats and this was associated with neutrophil recruitment in response to bleomycin (Serrano-Mollar et al., 2002). E-selectin is synthesised and expressed on endothelial cells in response to inflammatory cytokines such as tumour necrosis factor (TNF) or interleukin-1 (IL-1) (Rosen and Bertozzi, 1994; Lasky, 1995; Tedder et al., 1995). L-selectin is expressed constitutively on leukocytes, but its presentation at the cell surface may be regulated (Rosen and Bertozzi, 1994; Lasky, 1995; Tedder et al., 1995). The precise identities of the ligands for the three currently known selectins are still not clear and are a matter of some controversy (Varki, 1997).

1.6 Cell cycle control

The cell cycle control machinery is divided in four phases: G1 (gap 1), S (synthesis of DNA), G2 (gap 2) and M (mitosis) phase regulated by a complex network of cyclins and cyclin dependent kinase (CDK) (Pagano et al., 1994; Lane, 1998; Lane, 2001; Nigg, 2001; Bartek et al., 2001; Brooksbank, 2000; Grana and Reddy, 1995; Nigg, 1995; Elledge, 1996; Paggi et al., 1996; Beijersbergen and Bernards, 1996; Sherr, 1996; Yamauchi and Bloom, 1997; Nurse et al., 1998; Ekholm and Reed, 2000; Murakami and Nurse, 2000; Jones and Kazlauskas, 2001; Furukawa, 2002). A simplified diagram representing the cell cycle control is shown in Figure 1.7.

Cell-cycle progression in higher eukaryotic cells is regulated by the cyclin-dependent kinases (CDKs). G1 cyclins include D-cyclin (D1, D2 and D3), cyclin E, and their catalytic subunits CDKs, (cdk4, cdk6 or cdk2) play an essential role in the G1 to S phase transition (Reed et al., 1994; Mann and Jones, 1995; Deng et al., 1995; Linke et al., 1996; Krämer et al., 1996; Juan et al., 1998; Cayrol et al., 1998; Moll et al., 2000; Ekholm and Reed, 2000; Bartek and Lukas, 2001).

E2F binds to retinoblastoma (Rb) protein and its relatives p107 and p130 (Windle et al., 1990; Beijersbergen and Bernards, 1996; Baldi et al., 1996; Kinoshita et al., 1996; Paggi et al., 1996; Herwig and Strauss, 1997; Dosaka-Akita et al., 1997; Philips et al., 1997; Amellem et al., 1998; Garriga et al., 1998; Marchetti et al., 1998). The formed complexes are able to bind to E2F transactivation domains, repressing E2F dependent transcription (Mann and Jones, 1995; Shiyanov et al., 1996; Day et al., 1997; Yamasaki, 1999; Delavaine and La Thangue, 1999; Gartel et al., 2000; Jiang et al., 2000). Hyperphosphorylation of Rb by Cdk in late G1 disrupts the Rb-E2F complex and prevents the formation of complex with newly synthesised E2F molecules. Underphosphorylation of Rb is a negative regulation of DNA synthesis, making phosphorylation of Rb by Cdk a crucial event regulating restriction point (R-point) transit (Day et al., 1997; Medema et al., 1995; Beijersbergen and Bernards, 1996; Paggi et al., 1996; Garriga et al., 1998; Marchetti

et al., 1998). Phosphorylation of Rb in G1 is sufficient to allow cells to pass the R-point, but not sufficient to permit entry intro S-phase (Beijersbergen and Bernards, 1996; Paggi et al., 1996; Herwig and Strauss, 1997; Day et al., 1997). The E-type cyclins are the other important positive regulatory of transcriptional control and DNA replication since they activate the transcriptional factor E2F (Day et al., 1997; Yamasaki, 1999; Delavaine and La Thangue, 1999; Jiang et al., 2000).

In the S phase, the cyclin E is exchanged with cyclin A. The cyclin A-Cdk2 complex is needed in replication foci for DNA synthesis as well as suppression of E2F transcriptional activity by phosphorylating Rb in the complex with E2F (Mann and Jones, 1995; Shiyanov et al., 1996; Yamasaki, 1999). Cyclin A also binds with Cdk1 (Cdc 2) in the G2 phase and then is suddenly degraded (Tchou et al., 1996; Cayrol et al., 1998; Niculescu et al., 1998; Dulic et al., 1998; Grana and Reddy, 1995; Garriga et al., 1998). Entry into mitosis (M phase) is triggered by the Maturation Promoting Factor (MPF), which is composed of Cdk1 and Cyclin B (Grana and Reddy, 1995; Nigg, 1995; Elledge, 1996; Beijersbergen and Bernards, 1996; Sherr, 1996; Herwig and Strauss, 1997; Nurse et al., 1998; Jones and Kazlauskas, 2001). Cyclin B destruction is required for mitosis exit (King et al., 1994; Waldman et al., 1996; Dulic et al., 1998).

A number of cyclin-dependent kinase inhibitors (CKIs) play a major role in the control of the cell cycle machinery including p15, p16, p18, p21 and p27 (Pagano et al., 1994; Mann and Jones, 1995; Grana and Reddy, 1995; Nigg, 1995; Hangaishi et al., 1996; Elledge, 1996; Paggi et al., 1996; Beijersbergen and Bernards, 1996; Linke et al., 1996; Sherr, 1996; Gadbois and Lehnert, 1997; Somasundaram et al., 1997; Herwig and Strauss, 1997; Yamauchi and Bloom, 1997; Ji et al., 1997; Cai and Dynlacht, 1998; Nurse et al., 1998; Cayrol et al., 1998; Costanzi-Strauss et al., 1998; Niculescu et al., 1998; Ekholm and Reed, 2000; Jones and Kazlauskas, 2001; Bartek and Lukas, 2001). This discussion is focused upon the regulation of the cell cycle via p21.

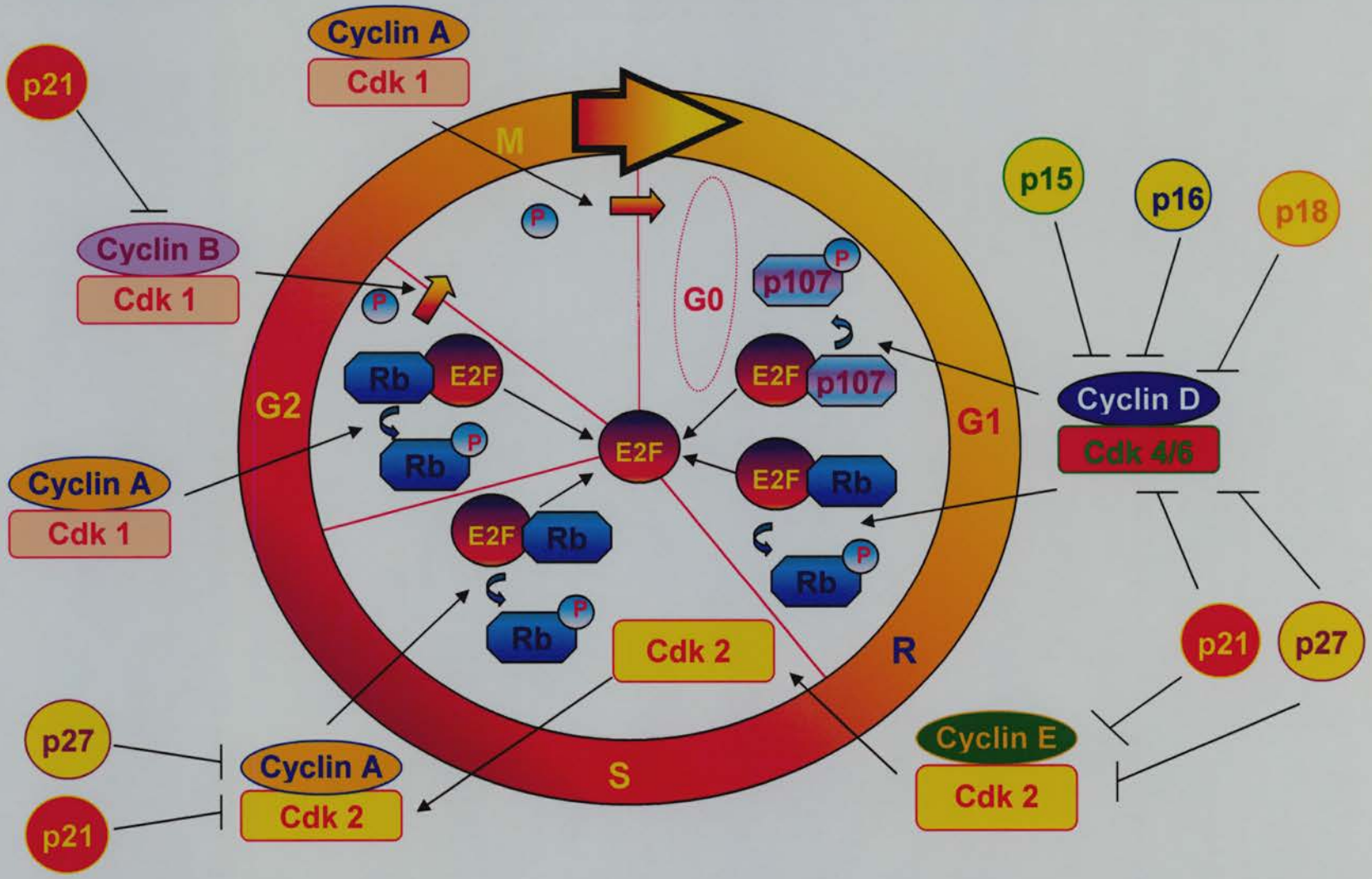


Figure 1.7 - Simplified diagram representing the regulation of cell cycle control. E2F (transcription factor) binds to retinoblastoma protein (Rb) and its relatives p107 and repress E2F dependent transcription. Hyperphosphorylation of Rb by cyclin-dependent kinase (Cdk) disrupts Rb-E2F complex. A number of cyclin-dependent kinase inhibitors (CDKI) play a major role in the control of cycle machinery including p15, p16, p18, p21 and p27.

1.7 Biology of p21

$p21^{WAF1/Cip1}$ belongs to the Cip/Kip family of cyclin kinase inhibitors (CKI) ($p21^{Waf1/Cip1}$, $p27^{Kip1}$, $p57^{Kip1}$). $p21^{Waf1/Cip1}$ was first described as a potent and universal inhibitor of cyclin-dependent kinases (Cdks) (Harper et al., 1993; El-Deiry et al., 1995; Ball, 1997). p21 functions as a checkpoint in the cell cycle by inhibiting cdks at the G1/S and G2/M interfaces (Zhang et al., 1993; Ji et al., 1997; Cayrol et al., 1998; Cai and Dynlacht, 1998; Bates et al., 1998; Niculescu et al., 1998; Dotto, 2000; Bartek and Lukas, 2001; Jones and Kazlauskas, 2001). p21 has been shown to bind to cyclin-cdk complexes, preventing phosphorylation of the retinoblastoma protein (Gartel et al., 1998; Costanzi-Strauss et al., 1998; Windle et al., 1990; Paggi et al., 1996; Herwig and Strauss, 1997; Yamauchi and Bloom, 1997; Day et al., 1997; Amellem et al., 1998; Garriga et al., 1998). When this happens the E2F pathway is blocked and the cell cycle is arrested at the G1/S interface (Cai and Dynlacht, 1998; Bates et al., 1998; Dotto, 2000; Delavaine and La Thangue, 1999; Gartel et al., 2000; Jiang et al., 2000).

Variation in the p21 levels has been shown in epithelial cells in several lung diseases (Takeshima et al., 1998; Kuwano et al., 1996; Rancourt et al., 2001; McGrath, 1998; Mishra et al., 2000; Kuwano et al., 2000; Gadbois and Lehnert, 1997; Guinee et al., 1996), upon cell-matrix disruption (Wu and Schönthal, 1997; Ilic et al., 1998; Howe, 2001; Vitale et al., 1999) and to be influenced by several integrins (Bachelder et al., 1999a; Clarke et al., 1995; Strömblad et al., 1996).

p21 expression is usually controlled at the transcriptional level by either the p53-dependent or -independent pathway (Michieli et al., 1994; Prost et al., 1998; Nurse et al., 1998; Murakami and Nurse, 2000; Zeng and El-Deiry, 1996), but p21 expression can also be regulated at the post-transcriptional level. Short wavelength UVC was found to induce p21 in a p53-dependent fashion by post-transcriptional modifications that led to enhanced stability of p21 mRNA (Gorospe et al., 1998; Wang et al., 2000). The transcription factors C/EBPα and C/EBPβ were found to interact with

p21 protein and protect it from proteolytic degradation (Timchenko et al., 1996; Timchenko et al., 1997; Krämer et al., 1996; Cram et al., 1998; Cortes-Canteli et al., 2002). In a number of other cell lines, post-transcriptional events strongly influence p21 expression following genotoxic stress (Butz et al., 1998). Stress-activated kinases p38α and JNK 1 were shown to stabilise p21 by phosphorylation at serine 130 (Kim et al., 2002).

p21 was shown to promote apoptosis (McKay et al., 1998; Gervais et al., 2000), protect cells from undergoing apoptosis (McKay et al., 1998; Lu et al., 1998; Bulavin et al., 1999; Asada et al., 1999; Zhang et al., 1999; Gervais et al., 2000), inhibit differentiation (Yamamoto et al., 1998; Harvat et al., 1998) and promote differentiation (Steinman et al., 1994; Liu et al., 1996a; Liu et al., 1996b; Billon et al., 1996; Matsumura et al., 1997; Nadal et al., 1997). p21 was also found to be present in the cytoplasm and various forms and sizes have been described (Orend et al., 1998; Asada et al., 1999; Poon and Hunter, 1998; Donato and Perez, 1998; Tchou et al., 1996; Gervais et al., 2000; Zhang et al., 1999; Jin et al., 2000).

1.7.1 Structure and protein interactions of $p21^{Waf1/Cip1}$.

Cip/Kip family ($p21^{Waf1/Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$ share significant sequence homology in their amino-terminal (Sherr, 1996; Nigg, 1995; Grana and Reddy, 1995; Elledge, 1996; Nurse et al., 1998). The amino-terminal domain of p21, like the corresponding domains of p27 or p57, is both necessary and sufficient to inhibit cyclin/CDK activity. The unique carboxy-terminal domain of $p21^{Waf1/Cip1}$ associated with the proliferating nuclear antigen (PCNA), a subunit of DNA polymerase δ and can inhibit DNA replication directly, without affecting DNA repair (Li et al., 1995a; Stillman, 1996; Cayrol et al., 1998; Oku et al., 1998; Rousseau et al., 1999).

$p21^{Waf1/Cip1}$ was identified as a mediator of p53-induced growth arrest (El-Deiry et al., 1993; El-Deiry et al., 1995) and a direct regulator of CDK activity (Harper et al., 1993; Elledge et al., 1996). $p21^{Waf1/Cip1}$ plays a critical role in the negative control of cell growth, and is generally up-regulated in cell arrest either by cell contact, serum deprivation, differentiation or senescence (Harper et al., 1993; Elledge et al., 1996; Elledge, 1996; Noda et al., 1994; Cai and Dynlacht, 1998; Dotto, 2000).

The human p21 gene consists of 3 exons (68, 450 and 1600 bp) but the first exon is not translated. The human p21 protein is 164 amino acids with a molecular mass of 21 kDa, and has been conserved during evolution (El-Deiry et al., 1993).

$p21^{Waf1/Cip1}$ interacts directly with cyclins through a conserved region close to the N-terminus (Cyc 1), however it has a second weak cyclin binding near its C-terminus region (Cyc 2), which overlaps with its PCNA binding region domain. Moreover, p21 has a separate cyclin-dependant kinase domain (Cdk) binding site in its N-terminus region (Chen et al., 1996) (Figure 1.8). For optimal cyclin-Cdk inhibition, the binding to this site as well as one of the cyclins is required. p21 was shown to compete with p107 and p130 for binding to cyclin/Cdks, and to disrupt already formed complexes among these molecules (Shiyanov et al., 1996).

p21 was found to cause repression of the E2F-dependent transcription possibly by direct association with E2F factor (Delavaine and La Thangue, 1999). This means that E2F could function as an anchor of p21, bringing it in juxtaposition with an E2F-dependent transcription initiation complex, thereby inhibiting its function (Dotto, 2000; Shiyanov et al., 1996; Delavaine and La Thangue, 1999; Gartel et al., 2000; Jiang et al., 2000).

p21 can bind to the N-terminus of c-Myc, to interfere with the c-Myc-Max association, but at the same time, the interaction between c-Myc and p21 can directly counteract p21-dependent inhibition of DNA synthesis, as c-Myc binds to the C-terminus of p21 in competition with PCNA (Kitaura et al., 2000)

p21 can enhance the function of transcription co-activators such as p300 (Perkins et al., 1997; Snowden et al., 2000). The ability of p300 to interact with NFκB-dependent transcription is negatively controlled by association of p300 with active cyclin/CDK complexes. Thus the inhibition of cyclin/CDK activity could be a way of explaining the capability of p21 to activate p300-dependent transcription. It was shown that stimulation of p300 activity could occur by p21 independently of the intrinsic histone acetyltransferase activity of this co-activator and its cyclin/CDK binding region. This occurs because p21 can relieve the effects of a previously uncharacterised transcription repression domain present in p300 (Snowden et al., 2000; Billon et al., 1996; Owen et al., 1998).

The E7 protein of human papilloma virus 16 (HPV-16) binds to p21 and competes with PCNA for binding to the carboxy-terminus domain of p21 (Funk et al., 1997; Jones et al., 1997). The association of E7 to p21, blocks the ability of p21 to inhibit cyclin/CDK activity as well as PCNA-dependent DNA synthesis. E7 does not disrupt the association of p21 with cyclin/CDK complexes, but it is thought to relieve p21-dependent suppression of CDK activity (Funk et al., 1997; Jones et al., 1997; Amellem et al., 1998).

Two DNA metabolising enzymes Fen 1 (Fap endonuclease I) and DNA-(cytosine-5) methytransferase (DNA MTase) bind to p21 in competition with PCNA. PCNA interacts physically with Fen I and stimulates its enzymatic activity. The central residues of the PCNA binding domain of p21 are the most highly conserved with Fen 1, and likely to mediate their mutually exclusive binding to PCNA (Li et al., 1995b; Wu et al., 1996; Warbrick et al., 1997). DNA MTase was shown to bind directly to PCNA and the PCNA-DNA MTase association was disrupted by p21. Newly replicated DNA must be methylated before histone H1 is incorporated into the nucleosome. This suggests that p21 can control DNA methylation levels during replication and possibly during DNA repair (Chuang et al., 1997).

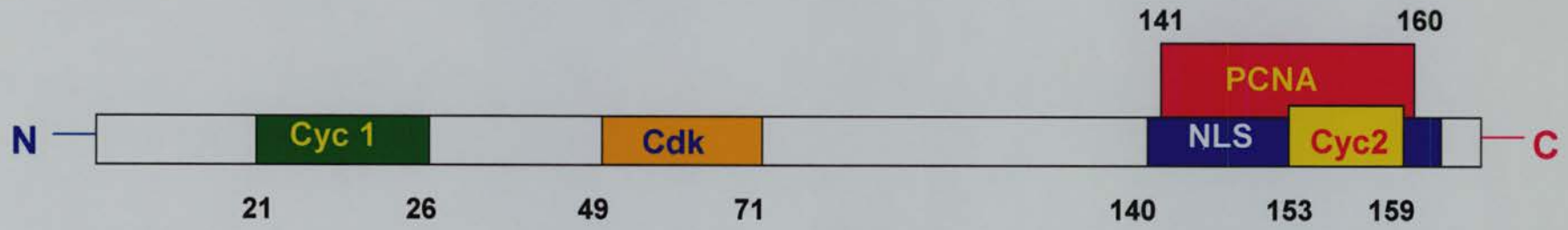
In addition to DNA metabolising enzymes, GADD45 is also involved in p21-PCNA interactions. GADD45, like p21 is a nuclear protein and has been implicated in induction of growth arrest, apoptosis, excision repair and DNA stability (Smith et al., 1994; Hall et al., 1995; Kearsey et al., 1995; Vairapandi et al., 1996; Hollander et al., 1999). Although GADD45 interacts with PCNA through a region distinct from that recognised by p21, both p21 and GADD45 compete with each other to bind PCNA (Smith et al., 1994; Hall et al., 1995; Kearsey et al., 1995). GADD45 and its related homologue MyD118, can directly associate with p21 (Kearsey et al., 1995; Vairapandi et al., 1996). GADD45 does not appear to inhibit cyclin-Cdk complexes either by itself or in combination of p21 (Smith et al., 1994).

p21 binds directly to procaspase 3 at its N-terminus and is involved in death induction and suppression. p21 also bind to SAPK at its N-terminus and was found to block its phosphorylation and also the activation by the upstream MKK4 kinase (Pochampally et al., 1999; Suzuki et al., 1999; Swanton et al., 1999).

p21 was shown to interact with other regulatory proteins, such as protein kinase, CK2 (Gotz et al., 1996), calmodulin (CaM) (Taules et al., 1999), a novel cell regulatory protein with oncogenic potential, SET (Estanyol et al., 1999), and the transcription factor C/EBP-α (Timchenko et al., 1997; Chinery et al., 1997; Nord et al., 1998).

The cell-cycle inhibitory activity of $p21^{Cip1/Waf1}$ is associated with its nuclear localisation. However a 10-amino acid truncation from the C-terminal form of p21 was reported in UV-irradiated normal diploid fibroblasts and many tumour cells mainly localised in the cytoplasm (Poon and Hunter, 1998).

A physical association between cytoplasmic p21 and ASK1 has been described and was found to suppress the activity of ASK 1 and MAPK (SAPK/JNK) cascade activation thus preventing the cell from undergoing apoptosis (Asada et al., 1999). It has been suggested that cytoplasmic p21 is formed by cleavage or truncation during apoptosis (Prabhu et al., 1997; Donato and Perez, 1998; Gervais et al., 2000; Jin et al., 2000; Zhang et al., 1999). Other forms of cytoplasmic p21 have been described but their roles are still unclear (Donato and Perez, 1998; Tchou et al., 1996; Gervais et al., 2000; Orend et al., 1998; Zhang et al., 1999; Jin et al., 2000).



E2F (1 – 90)

procaspase 3 (1 – 33)

SAPK (1 – 64)

ASK 1 (1 – 140)

c-Myc (139 – 164)

HPV-16 E7 (139 –164)

GADD45 (139 – 164)

Calmodulin (145 – 164)

SET (145 – 164)

C/EBP-α (~84 – 164)

Figure 1.8 – Map of the structure p21$^{WAF1/Cip1}$ protein showing the N- and C- terminus domains and of its direct protein-protein interactions. Cyc 1 and 2 are the cyclin binding domains, NLS is the nuclear localization signal, PCNA is proliferating cell nuclear antigen, and Cdk is cyclin-dependent kinase. Adapted from (Dotto, 2000).

1.7.2 p53-dependent induction of p21 transcription.

The p53 tumour suppressor protein is an inducible transcription factor required for the transactivation of a number of genes involved in cell cycle control (Elledge, 1996; Lane, 1992; Vogelstein et al., 2000; Paggi et al., 1996; Sherr, 1996; Nurse et al., 1998; Ekholm and Reed, 2000; Jones and Kazlauskas, 2001). p21 expression is normal in embryos and most tissues from p53 knockout (p53 -/-) mice (Macleod et al., 1995).

Although p53 is not required for p21 transcription, the regulation of p21 is p53-dependent following DNA damage by γ-radiation (Macleod et al., 1995). Cultured p21-deficient mouse embryonic fibroblasts still had the ability to undergo G1 arrest in response to DNA damage, thus p53 may induce an additional gene that participates in cell arrest (Deng et al., 1995; Brugarolas et al., 1995). p21 transcription and cell cycle arrest was observed in irradiated human cell lines (Dulic et al., 1994; Namba et al., 1995).

The expression of p21 was found to increase via the p53-dependent pathway in murine embryonic fibroblasts following treatment with the spindle disrupter nocodazole (Lanni and Jacks, 1998). Ribonucleotide biosynthesis inhibitors such as pyrazofurin and cyclopentenylcytosine were shown to induce p53-dependent p21 expression in the absence of DNA damage, and resulted in hypophosphorylation of retinoblastoma in normal human fibroblasts and cell growth arrest (Linke et al., 1996).

Transcriptional induction of p21 by p53 may prevent cells undergoing apoptosis and instead lead to cell-cycle arrest (Polyak et al., 1996). A summary of the induction of p21 via the p53-dependent pathway and control of apoptosis via p21 is shown in figure 1.9.

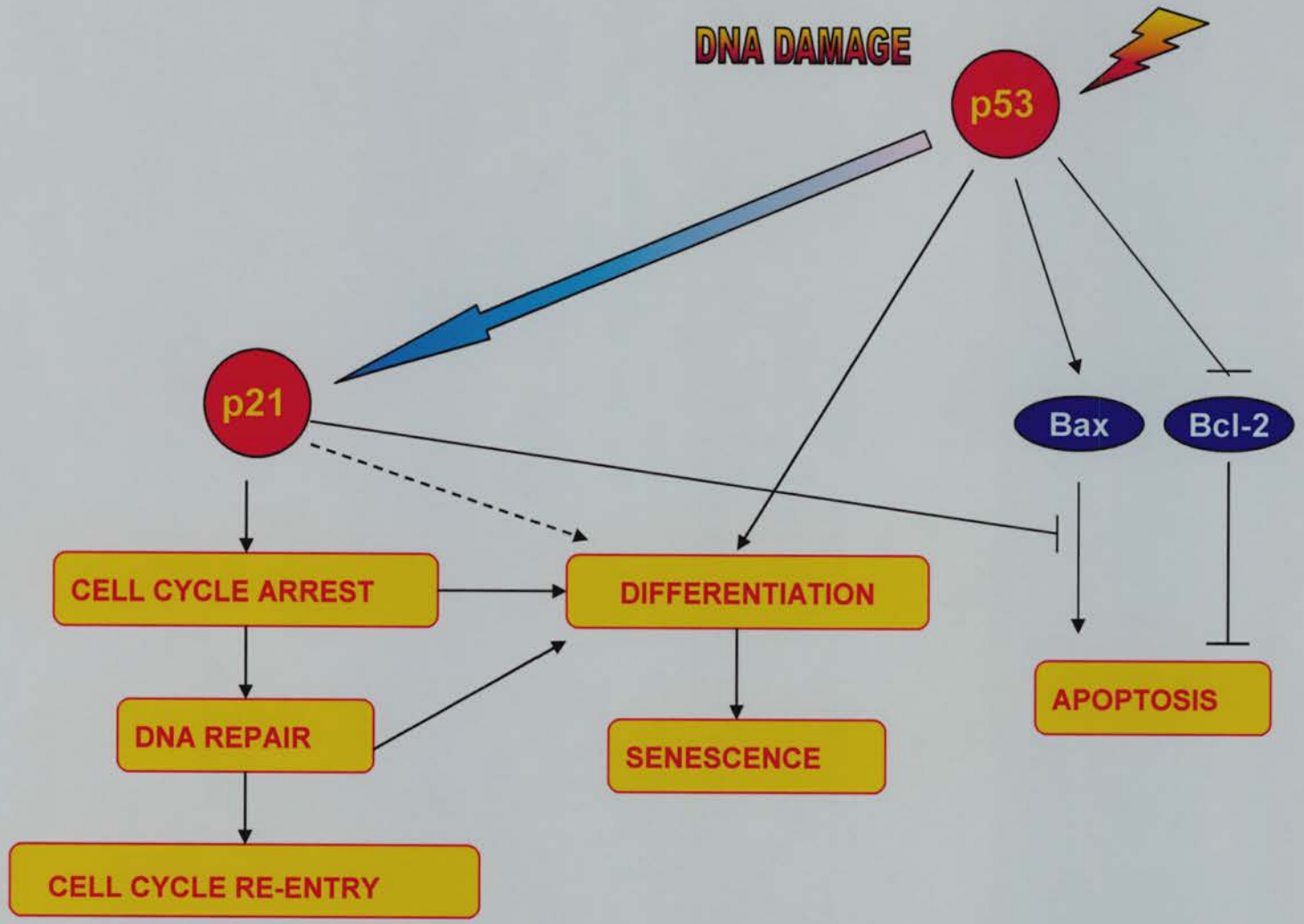


Figure 1.9 - p21 regulation via the p53 dependent pathway. p53 induction may lead cells down the apoptotic pathway, by upregulating Bax and downregulating a survival factor, Bcl-2. Transcriptionally induced p21 by p53 may prevent this and instead lead to cell cycle arrest. Adapted from (Cox, 1997).

1.7.3 p53-independent regulation of p21 expression.

A number of agents activate p21 transcription independent of the p53 pathway. These agents induce binding of different transcription factors to specific cis-acting elements located within the p21 promoter (Table 1.4).

The region between –119 bp and start of transcription of the human p21 gene contains six Sp1 binding sites (Sp1-1 to Sp1-6). Sp1 is a member of a multigene family that binds to DNA through C-terminal zinc-finger motifs. Sp2, Sp3 and Sp4 share extensive structural and sequence homology with Sp1 (Kennett et al., 1997).

Phorbol ester (PMA) and okadaic acid induce p21 through Sp1 (Kennett et al., 1997; Biggs et al., 1996). The tumour suppressor protein BRCA1 activates p21 via the region from –143 to –93 bp which contains the Sp1-1 and Sp1-2 sites and inhibits DNA synthesis (Somasundaram et al., 1997). Transforming growth factor-β (TGF-β) (Datto et al., 1995; Li et al., 1998), calcium (Prowse et al., 1997), butyrate (Nakano et al., 1997), lovastatin (Lee et al., 1998), histone deacetylase inhibitor Trichostatin A (TSA) (Sowa et al., 1997; Nakano et al., 1997) and nerve growth factor (NGF) (Billon et al., 1996) has been shown to induce p21 via the Sp1-3 site in the p21 promoter. TGF-β and butyrate inhibited proliferation and induced G1 cell cycle arrest in various cells (Datto et al., 1995; Li et al., 1998; Sowa et al., 1997; Nakano et al., 1997), calcium induced differentiation of cultured mouse keratinocytes (Prowse et al., 1997) whereas lovastatin induced cell cycle arrest in p53-null human prostate carcinoma cells (Lee et al., 1998).

The addition of nerve growth factor (NGF) to PC12 cells induced p21 expression and differentiation through Sp1, Sp3 sites and p300 transcriptional co-activator (Billon et al., 1996; Yan and Ziff, 1997). Progesterone was found to increase the level of p21 and promote growth arrest. The progesterone receptor (PR) was found complexed with p300 and Sp1 (Owen et al., 1998).

A variety of other transcription factors such as AP2 (Zeng et al., 1997), E2F (Mann and Jones, 1995; Shiyanov et al., 1996; Yamasaki, 1999; Delavaine and La Thangue, 1999; Gartel et al., 2000), STATs (Chin et al., 1996; Grandis et al., 1998; Lee et al., 1998; Coqueret and Gascan, 2000) and C/EBPα (Chinery et al., 1997; Nord et al., 1998) can induce p21 transcription in response to different signals (Table 1.4).

p21 inducing agents	Transcription factors mediating p21 gene expression	cis-acting elements	References
Butyrate	Sp1, Sp3	-82 /-77 bp; -69/-64 bp	(Nakano et al., 1997)
Lovastatin	Sp1, Sp3	-82 /-77 bp	(Lee et al., 1998)
Trichostatin A	Sp1, Sp3	-82 /-77 bp; -69/-64 bp	(Sowa et al., 1997)
TGF-β	Sp1, Smad 3/4, Smad 2/4, p300	-82 /-77 bp	(Datto et al., 1995; Li et al., 1998; Moustakas and Kardassis, 1998)
NGF	Sp1, Sp3, p300	-82 /-77 bp	(Billon et al., 1996)
Calcium	Sp3	-82 /-77 bp	(Prowse et al., 1997)
Progesterone	Sp1, p300	-82 /-77 bp; -69/-64 bp	(Owen et al., 1998)
Okadaic acid	Sp1, Sp3(?)	-119/-114 bp; -109/-104 bp; -82/-77 bp; -69/-64 bp	(Biggs et al., 1996)
	Ap2	-102/-94 bp	(Zeng et al., 1997)
TPA	Ap2	-102/-94 bp	(Zeng et al., 1997)
PMA	Sp1, Sp3(?)	-119/-114 bp; -109/-104 bp	(Biggs et al., 1996)
-	BRCA1	-143/-93 bp	(Somasundaram et al., 1997)
-	E2A (E47)	-162/-157 bp; -20/-15 bp; -5/-1 bp	(Prabhu et al., 1997)

p21 inducing agents	Transcription factors mediating p21 gene expression	cis-acting elements	References
Vitamin D3	VDR	-779/-765 bp	(Liu et al., 1996b)
Retinoic acid	RAR	-1212/-1194 bp	(Liu et al., 1996a)
Dexamethasone	C/EBPα	-1270/-1256 bp	(Cram et al., 1998)
Vitamin E	C/EBPα	-1928/-1920 bp	(Chinery et al., 1997)
EGF	STAT 1, STAT 3	-4236/-4288 bp; -2561/-2553 bp; -696/-688 bp	(Chin et al., 1996)
IFN-γ	STAT 1	-4236/-4228 bp; -2561/-2553 bp; -696/-688 bp	(Chin et al., 1996)
TPO	STAT 5	-4236/-4228 bp; -2561/-2553 bp;	(Matsumura et al., 1997)
IL6	STAT 3	-696/-688 bp	(Bellino et al., 1998)

Table 1.4 – p21 inducing agents that activate p21 transcription independent of p53 and their specific cis-acting elements located within the p21 promoter. Adapted from (Gartel and Tyner, 1999).

1.7.4 p21 and differentiation

Exit from the cell cycle is a prerequisite for terminal differentiation, and p21 expression is induced during terminal differentiation both *in vitro* and *in vivo* (Michieli et al., 1994; Steinman et al., 1994; Halevy et al., 1995; Missero et al., 1995). p21 expression promotes differentiation in megakaryoblast leukemia cell line (CMK) (Matsumura et al., 1997), in megakaryocytes cells ($CD34^+$) (Baccini et al., 2001), in myelomonocytic cell line (U937) (Liu et al., 1996a; Liu et al., 1996b), in chondrosarcoma cells (SW1353) (Hiraoka et al., 2002), in dendritic cells and macrophages from human peripheral blood monocytes (Kramer et al., 2002), in skeletal muscles (Park and Chung, 2001), in mice lungs (Park and Chung, 2001), in peripheral nervous system neurons in response to nerve growth factor mediated by p300 protein (Billon et al., 1996), in myotube differentiation from myoblasts under the influence of the transcription factor MyoD (Halevy et al., 1995) and in laryngeal tumours (Nadal et al., 1997). Interestingly, p21 was also shown to inhibit differentiation in terminally differentiated of mouse keratinocytes (Di Cunto et al., 1998) but not those of humans (Harvat et al., 1998) and decrease differentiation in human colon cancer cell line HT29 (Yamamoto et al., 1998). The p21 was found not to be involved in the regulation of differentiation of the mouse skin tumours (Weinberg et al., 1999) and in mouse keratinocytes (Weinberg et al., 1997).

In the p21 null mice, normal differentiation has been observed thus implying that p21 is not a mutually exclusive agent that promotes differentiation (Paramio et al., 2001; Cox, 1997; McDonald et al., 1996; Deng et al., 1995). Various other genes are thought to be involved and maybe cooperate with p21 to regulate differentiation including p15 (Paramio et al., 2001), p16 (Harvat et al., 1998; Paramio et al., 2001; Furukawa, 2002), p18 (Paramio et al., 2001), p19 (Paramio et al., 2001), p27 (Yamamoto et al., 1998; Harvat et al., 1998; Furukawa, 2002; Deschenes et al., 2001), p53 (Soddu et al., 1996; Macleod et al., 1995; Nadal et al., 1997; Xue et al., 2002), p57 (Dyer and Cepko, 2000; Park and Chung, 2001; Nakai et al., 2002) and Rb (Baccini et al., 2001; Deschenes et al., 2001; Xue et al., 2002).

1.7.5 p21 and proliferation

p21 is usually assumed to inhibit proliferation both *in vitro* and *in vivo* (Balomenos et al., 2000; Sugibayashi et al., 2002) and by the introduction of p21 expression constructs into normal (Harper et al., 1993) and tumour cell lines (El-Deiry et al., 1993) resulted in cell cycle arrest at the G1 phase of the cell cycle (Harper et al., 1995). Paradoxically, p21 has been shown to promote proliferation under certain circumstances (Mantel et al., 1996; Lai et al., 2002).

Various mechanisms have been described by which p21 can regulate proliferation (Niculescu et al., 1998). p21 may induce growth arrest by inhibiting the activity of cyclin-dependent kinases (Cdks) or of proliferating cell nuclear antigen (PCNA) (Kelman, 1997). The unique carboxy-terminal domain of p21 can associate with PCNA and DNA polymerase δ and ε and can inhibit DNA replication directly, without affecting DNA repair (Chen et al., 1996; Mantel et al., 1996; Oku et al., 1998; Li et al., 1995a; Warbrick et al., 1997; Funk et al., 1997; Chuang et al., 1997; Cayrol et al., 1998; Rousseau et al., 1999; Li et al., 1994; Shivji et al., 1994). p21 may act as an assembly factor for Cdk/cyclin complexes. p21 was found to promotes the assembly of Cdk4/6 and cyclin D *in vitro* and was found associated with cyclinD/Cdk4 complexes during cell-cycle progression (LaBaer et al., 1997; Morisaki et al., 1999; Cheng et al., 1999; Wong et al., 2001). p21 was also shown to complex with cdk2 and thus lead to growth arrest (Niculescu et al., 1998; Shiyanov et al., 1996; Jones et al., 1997; Orend et al., 1998; Morisaki et al., 1999; Aikawa et al., 2001). The role of p21 as an assembly activator or inhibitor depends on its expression level. At low and intermediate concentrations it is an assembly factor, while at high concentrations it is an inhibitor (LaBaer et al., 1997; Hiyama et al., 1997; Morisaki et al., 1999).

p21 has been shown to be part of a quaternary complex that contains cyclin, Cdk and PCNA (Zhang et al., 1993). The transition between active and inactive states occurs through changes in p21 stoichiometry, particularly when multiple p21 molecules

versus a single p21 molecule bind to these complexes (Harper et al., 1995; Zhang et al., 1994).

The role of p21 to either promote or inhibit proliferation could depend on the specific cellular context (Bearss et al., 2002). Mammary tumour-susceptible MMTV-ras mice displayed higher S-phase fractions and an increase in tumours in p21 knockout mice, whereas MMTV-c-Myc exhibited lower S-phase and a decrease in tumours (Bearss et al., 2002).

Summary of the regulation of proliferation by p21 is shown in Figure 1.10.

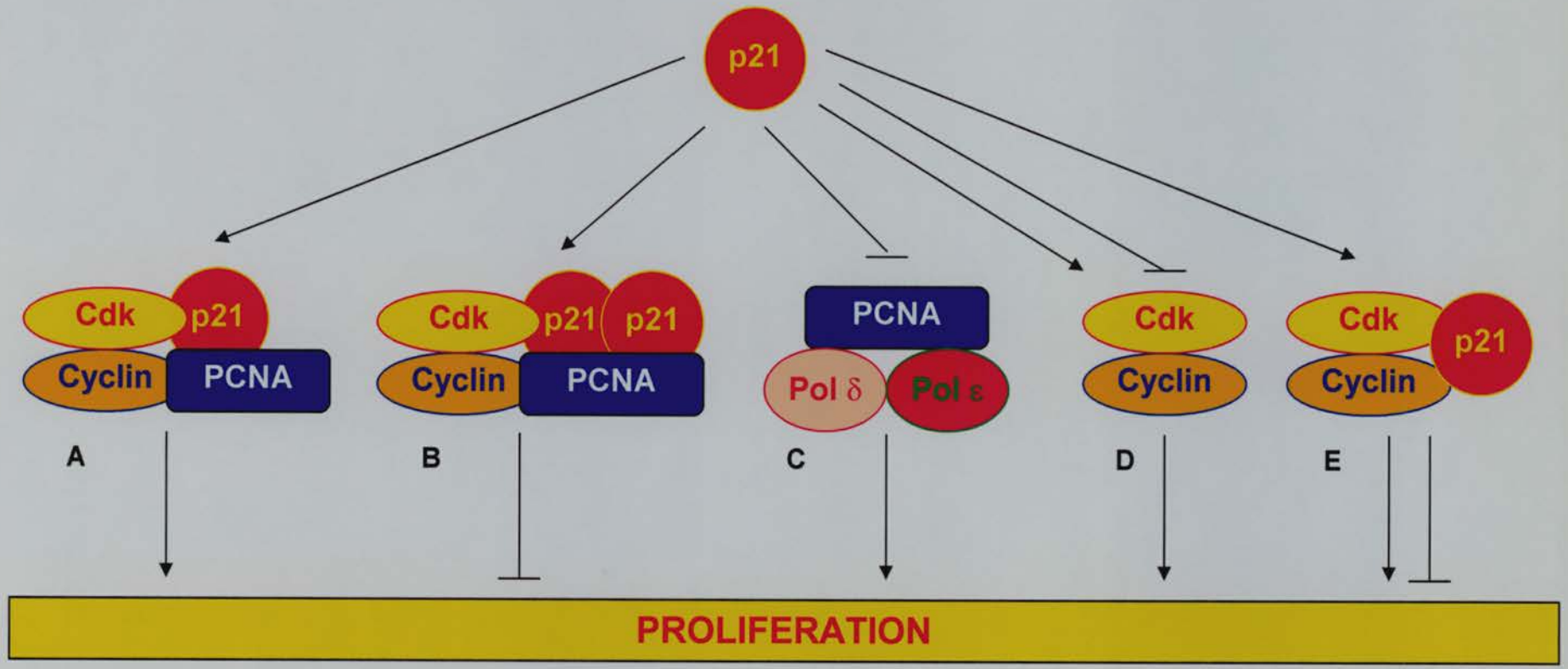


Figure 1.10 - Regulation of proliferation by p21. (A) p21 can form a complex with Cdk, Cyclin and PCNA. (B) At low or intermediate conc. of p21 it promotes proliferation while at high p21 conc. the complex is inactive thus inhibiting proliferation. (C) p21 can inhibit the activity of PCNA complex with DNA polymerase δ and ε and (D) Cdk/Cyclin complex or act as an assembly factor for Cdk/Cyclin complexes. (E) p21 can also associate with the Cdk/Cyclin complexes and can inhibit or promote proliferation.

1.7.6 p21 and apoptosis

p21 can both promote and inhibit apoptosis although it generally counteracts the apoptotic process (Dotto, 2000). p53-dependent apoptosis occurs normally in cells from p21 knockout mice (Waldman et al., 1996). Expression of p21 appears to protect colorectal carcinoma, and melanoma cells from p53-induced apoptosis (Polyak et al., 1996; Gorospe et al., 1998; Wang et al., 2000; Gorospe et al., 1997; Mahyar-Roemer and Roemer, 2001), while suppression of p21 expression by anti-sense technology and homologous recombinations was shown to shift cells from the cell-cycle arrest pathway to apoptosis (Polyak et al., 1996). While the majority of evidence supports a role of p21 in protection against apoptosis, over-expression of p21 has been associated with the induction of apoptosis in human retinoblastoma cells lines (Kondo et al., 1997) and T-cells (Fotedar et al., 1999).

Cells from p21 knockout mice were shown to contain very high levels of apoptosis after γ-irradiation (Deng et al., 1995; Alan Wang et al., 1997; Bulavin et al., 1999). These findings might suggest that p21 protein normally protects cells from undergoing p53-mediated apoptosis by holding them in cell-cycle arrest (Cox, 1997; Deng et al., 1995; Alan Wang et al., 1997) (Figure 1.9).

Activation of the MAPKs of the SAPK (JNK) and p38 kinase families are also key events in the apoptotic response of many cells and p21 has been found to associate and control both types of molecules (Shim et al., 1996; Kim et al., 2002; Bacus et al., 2001). p21 was also found to form complexes with caspase 3 (Pochampally et al., 1999; Suzuki et al., 1998; Bachelder et al., 1999b; Suzuki et al., 1999; Swanton et al., 1999; Jin et al., 2000), and MEKKs (ASK1) (Asada et al., 1999). The association with these molecules could be favoured by the fact that p21 itself is a caspase substrate, and becomes localised to the cytoplasm as a consequence of caspase-dependent cleavage of its nuclear localisation C-terminus domain and is unable to suppress growth as well as apoptosis (Gervais et al., 2000; Levkau et al., 1998b; Park et al., 1998). This truncation would also compromise the ability of p21 to promote

cyclin/CDK nuclear localisation, with the same biological end point effect (Gervais et al., 2000).

p21 can also protect cells from undergoing apoptosis due to the cyclin/CDK inhibition (Sekiguchi and Hunter, 1998; Wu et al., 1998a; Jin et al., 2000; Wong et al., 2001; Harper et al., 1993; Zhang et al., 1994; Harper et al., 1995; De Luca et al., 1996; Chin et al., 1996; Chen et al., 1996; Sheikh et al., 1997; Ball, 1997; Owen et al., 1998; Morisaki et al., 1999; Rousseau et al., 1999). Direct interaction between p21 and GADD45 is thought to promote apoptosis rather than cell cycle arrest (Kearsey et al., 1995; Vairapandi et al., 1996; Hollander et al., 1999).

Two novel proteins, p21B and p21C, which are variants of p21 were found to be induced by DNA damage, p53 and p73. p21B have two unique exons and encodes a protein that is not homologous to p21 or any other known protein. p21B was found to induce cell cycle arrest and apoptosis. p21C uses an extended version of the p21B exon I, but is spliced to the second and third exons of p21 and encodes the p21 protein (Nozell and Chen, 2002).

Summary of the regulation of apoptosis by p21 is shown in Figure 1.11.

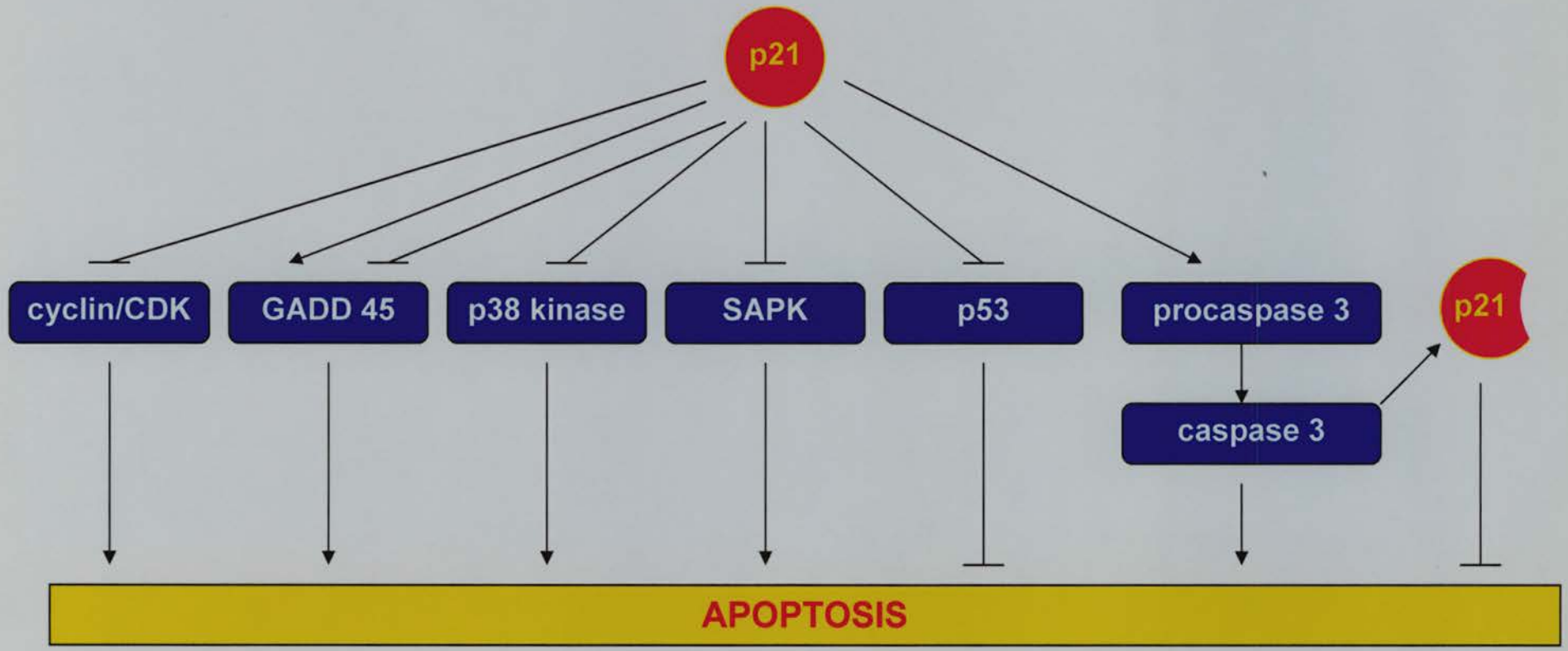


Figure 1.11. Regulation of apoptosis by p21. p21 can inhibit apoptosis by complexing with cyclin/CDK, Gadd45, p38 kinase, SAPK and can inhibit the p53-dependent apoptosis pathway. p21 can also regulate Gadd45 and procaspase 3 leading to apoptosis. Caspase 3 can truncate nuclear p21 at the C-terminal thus losing the nuclear localisation signal, and is located in the cytoplasm, whereby it cannot suppress growth arrest as well as apoptosis.

1.8 Hypothesis and Aims

After lung injury, Clara cells are damaged and may be shed thus disrupting the cell-cell and cell-matrix interaction and also denuding the basement membrane. The normal response to injury is usually an inflammatory reaction and the deposition of new extracellular matrix that incorporates various growth factors and inflammatory cells that help to regenerate the lost epithelial cells. Regeneration is carried out by migration, proliferation and differentiation of stem cells to cover the exposed area.

The main hypotheses of this thesis are:

1. p21 is a critical regulator of Clara cell proliferation, differentiation and death.
2. Clara cell response to changes in the environment is through integrin mediated changes in p21.

The main aims of this thesis are:

1. Describe what is happening in terms of cell cycle control in animal mouse models of lung injury.
2. To isolate, culture and characterise murine Clara cells.
3. Study the effect of extracellular matrix (ECM) and integrins on Clara cell proliferation, differentiation and death through p21 regulation.
4. Investigate the regulation of the cyclin kinase inhibitor $p21^{Cip1/Waf1}$ in Clara cells.

Chapter 2 - Materials and Methods

All materials, glassware and plasticware used throughout this project were sterile to prevent contamination or degradation of nucleic acid or proteins. Gloves were worn at all times. All procedures involving culturing were carried out using aseptic techniques. Before working with animals the author had to undergo training for small animal handling as required by the Scientific Procedures Act 1986. A cetificate was issued by ScotPil Training Committie in 1999, certificate number SCT-E99016. Since this work involved the use of transgenic animals, the auther had to undergo a Genetic Modification Traning couse organised by the Health and Safety Department, Univerisity of Edinburgh. All the procedures were assessed and appropriate COSSH forms signed.

Receipts of buffers and solutions that form part of kits are given in the text. Receipes for buffers and solutions that do not form part of kits are given in Appendix 1. Names of manufacturers are given in brackets. Full postal address of suppliers is given in Appendix 2.

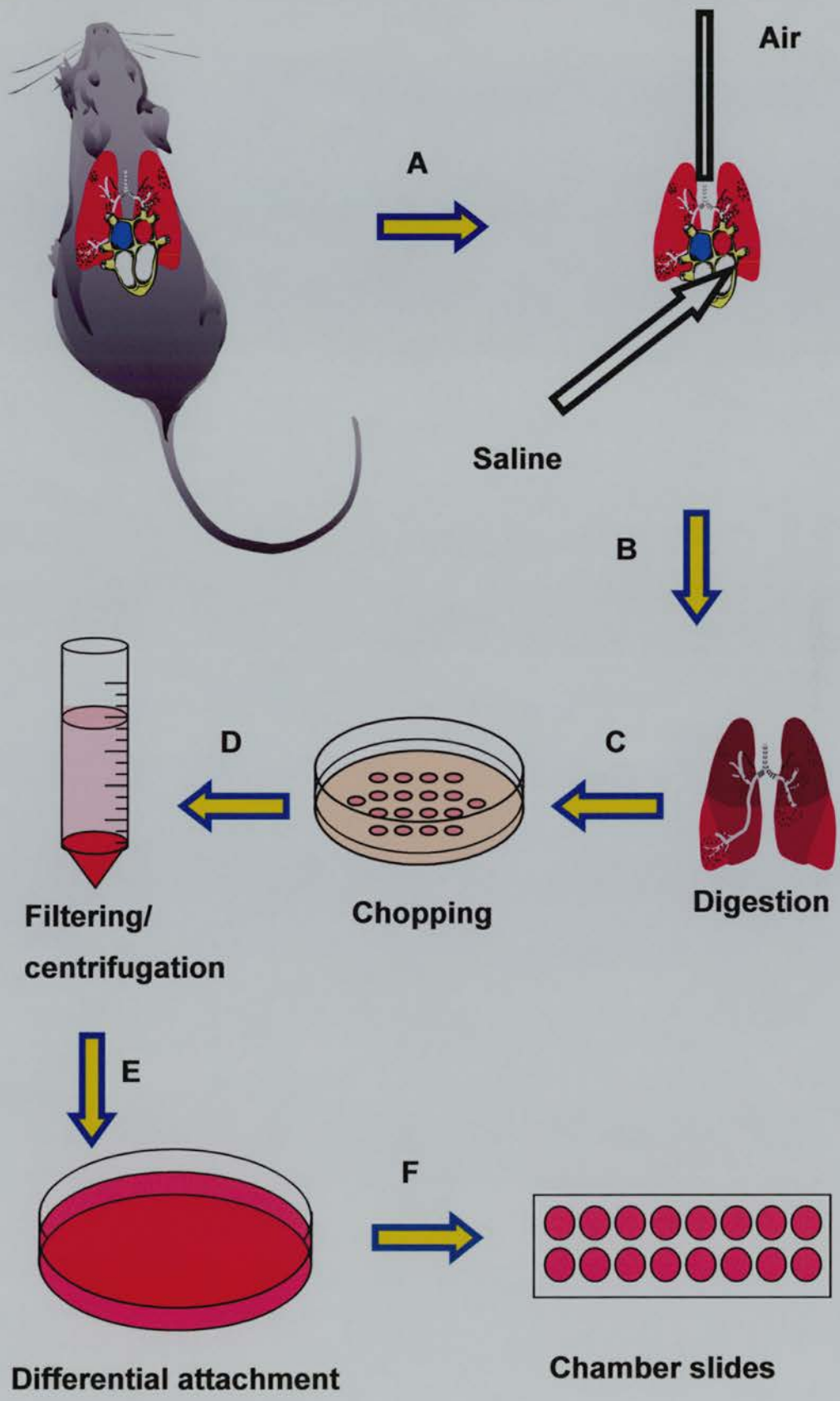
2.1 Primary Clara cell culture

2.1.1 Isolation and culturing of mouse bronchiolar cells.

Clara cells were isolated using a modification of methods described previously. (Belinksy et al., 1995; Van Scott et al., 1987; Masek and Richards, 1990; Richards et al., 1990; Oreffo et al., 1990). The procedure used here has been previously reported (McBride et al., 2000) and differs from that of Masek and Richards, (1990) as indicated in sections 2.1.2, 2.1.3 and 2.1.4. The procedure used to isolated Clara cells from p21 ko mice was the same used for wt mice. Summary of the isolation procedure is given in Figure 2.1.

Figure 2.1 – Clara cell isolation procedure.

A. Mice were sacrificed and dissected. Cannula was inserted inside the trachea
B. Lungs were perfused by inflating the lungs with air through the cannula and by introducing sterile saline into the right ventricle of the heart
C. Lungs were removed from the animal and trypsin was instilled into the lung. Lungs were incubated at 37°C for 15 minutes and chopped with scissors.
D. Cell suspension was filtered through 150 μ and 30μ nylon filters, washed with DNase I solution and centrifuged at low speed (32g).
E. Cells were transferred to a sterile petri-dish and incubated for 2 hours at 37°C. Macrophages and fibroblasts were removed by differential attachment.
F. Clara cells were collected by centrifugation, culture medium was added and cells were seeded to chamber slides.


Air
A
Saline
B
D
C
Filtering/
centrifugation
Chopping
Digestion
E
F
Differential attachment
Chamber slides

2.1.2 Dissection and lung perfusion.

Mice (C3H/HE strain or p21 ko mice either male or female, between 4 to 8 weeks old) were sacrificed by lethal intraperitoneal injection of 0.5ml pentabarbitone (Sagatal^{TM}). In this thesis about 3000 mice were used, approximately 1000 of which were p21 ko mice (section 2.4.1). The ventral surface skin was removed and a midline incision was made to allow entry into the peritoneal cavity. The gastrointestinal tract was displaced to the right and the major dorsal blood vessels cut. The position of the trachea was located, and a cannula (1mm Luer cannula, CAN1004, Scientific Lab. Supplies) was inserted from a partial cut at the top of the trachea. The diaphragm was carefully opened by a small incision just below the xiphisternum following by widened this cut in both directions. The rib cage was removed and care was taken not to puncture the lungs. A portion of the thymus was removed for easier access to the vessels around the heart.

The lower region of the heart was gripped with a haemostat, and a cannula (1.7mm Luer cannula, CAN1008, Scientific Lab. Supplies) was inserted into the pulmonary artery through an incision in the pericardium. Sterile saline (0.15M NaCl) was gravity fed through the cannula, causing an increase in size of the left atrium. An incision was made in the left atrium to allow fluid exit. Lungs were artificially ventilated with a 1.0ml syringe of air. After 3 ventilations, the lungs were totally free of blood and appeared completely white. The heart was detached and discarded.

2.1.3 Lung digestion.

A syringe containing preheated trypsin at 37°C was attached to the cannula and trypsin (T8003, Sigma) was intratracheally instilled. The lungs were detached at the bottom by cutting the oesophagus, the posterior vena cave and any strands of tissue holding them to the diaphragm and then the whole preparation was turned around so as to work from the top of the trachea. The lung was freed and removed intact from

the cavity. The lung was cleaned from any remains of oesophagus and any other debris. The lung was transferred into a vial containing preheated PBS at 37°C and incubated for 15 minutes at 37°C.

2.1.4 Cell isolation and purification.

From the point onwards the isolation was carried out in a sterile laminar flow tissue culture hood (Class II) using aseptic techniques and sterile solutions. Digested lungs from 8 mice were place in a sterile plastic petri dish and chopped with sterile scissors to small pieces of about 1mm. Foetal bovine serum (2mls/lungs) was added to the minced lungs to inhibit further trypsin activity. The minced lungs were transferred to a 50ml centrifuge tube and DNase I solution (3 mls/lungs) was added. Treatment the DNase I reduce viscosity. The mixture was hand-shaken for 4 minutes. The suspension was filtered through 150 μ and 30 μ nylon filters and topped-up to 50ml with DNase 2 solution. The tube was centrifuged at low speed 32g at 10°C for 6 minutes in order to obtain functionally active Clara cells. The supernatant was removed and 50 ml of DNase 2 solution was added to the pellet at the bottom of the tube. The tube was hand-shaken for 1 minute and centrifuged at 32g at 10°C for 6 minutes.

The supernatant was removed and 10 ml medium (1:1 M199/HamF12, 1% glutamine, 1% streptomycin) was added and hand-shaken for 1 minute. At this point the isolation consists predominantly of bronchiolar cells, macrophages and fibroblasts. The latter two cell types have an increased capacity for adherence than epithelial bronchiolar cells. The suspension was transferred to a sterile non-tissue culture petri-dish and incubated at 37°C for 2 hours at 5%CO_2/air to allow differential attachment.

The suspension was transferred to a 50 ml centrifuge tube and centrifuged at 100g for 5 minutes at 10°C. The supernatant was removed and appropriate culture medium (section 2.1.6) was added to an approximately 1 x 10^4 clumps/ml. 50μl aliquots were taken for an approximate cell count.

2.1.5 Cell culture

Cells were counted using a haemocytometer. However, this apparatus if designed primarily for quantification of single cell suspension. Isolated Clara cells are usually in clumps and with typical 'bunch of grapes' morphology. Thus for cell estimation, the number of clumps and individual cells were recorded and an estimate made of total cell number.

Once isolated, cells were either cytospun onto glass slides (time 0 hours) at 100 g for 6 minutes and then fixed with methanol at –20°C or plated onto 16-well glass chamber slides (Gibco) which had been pre-coated with appropriate ECM (section 2.1.7) and incubated at 37°C, 5% CO_2/air. Cells were allowed to attach overnight after which the medium was replaced to remove dead and unattached cells. Medium was subsequently replaced every 2 days. Cells were fixed at time 24, 72 and 120 hours by methanol at –20°C.

2.1.6 Culture medium.

Mouse bronchiolar cells were cultured in a 1:1 mixture of Hams F12 (Gibco) and M-199 medium (Gibco). The medium was supplemented with 2 mM L-glutamine, 10 μg/ml insulin, 5 μg/ml transferrin, 100 ng/ml hydrocortisone, 10 ng/ml EGF, 0.1 ng/ml retinyl acetate and Pen/Strep. (Belinksy et al., 1995; Van Winkle et al., 1996a; Richards et al., 1990; Oreffo et al., 1990; McBride et al., 2000).

2.1.7 Extracellular matrix (ECM) coated chamber slides.

16-well chamber slides (wells having 6mm diameter) (Gibco) were coated with 50μg of ECM overnight at 4°C. The next morning the chamber slides were washed with sterile PBS and stored and -20°C. Three different types of ECM components were

used in this study: fibronectin (Fn) (Sigma), collagen IV (Coll IV) (Sigma) and laminin (Lam) (Sigma). Fibronectin was used for the basal culture conditions of all cultures. Seven variations of ECM were used for further studies as shown in Table 2.1

Extracellular matrix (ECM) combinations	Concentration
Lam/Fn/Coll IV	50μg/ml of each added simultaneously
Coll IV/Fn	50μg/ml of each added simultaneously
Coll IV/Lam	50μg/ml of each added simultaneously
Lam/Fn	50μg/ml of each added simultaneously
Coll IV	50μg/ml
Lam	50μg/ml
Fn	50μg/ml

Table 2.1 - Different ECMs combinations and the concentrations upon which Clara cells were cultured.

2.2 Cell Biology

2.2.1 Tissue Sections

Lung tissues were fixed in buffered formalin for 16 hours and then processed to paraffin wax in vacuum impregnating processor. Paraffin emebedded sections were cut on a microtone to a thickness of 3μm.

2.2.1.1 De-waxing and re-hydration of slides

Paraffin tissue sections were place in xylene for 15 minutes. The sections were then transferred in a series of alcohol gradients from 100% to 74% to 64% and then tap water for 10 minutes each.

2.2.1.2 Antigen Retrieval

2.2.1.2.1 Trypsin antigen retrieval

A glass slide container was pre-warmed in 37°C incubator. Trypsin solution (100mg trypsin, 100 mg calcium chloride, 100 ml H_2O, adjusted to pH 7.6 using 0.2 M Tris) was pre-warmed for 5 – 10 minutes in a 37°C in a water bath. Slides that were de-waxed and rehydrated (as described above) were placed in glass slide container containing the pre-warmed trypsin for 30 minutes at 37°C. Then slides were rinsed with H_2O and equilibrated in TBS.

2.2.1.2.2 Microwave antigen retrieval

DAKO antigen retrieval solution was prepared by dilution 1:100 with distilled water. A microwave dish was filled up with antigen retrieval solution and pre-heated in a microwave to boiling point. Slides that were de-waxed and rehydrated (as described above) were placed in a plastic slide rack and placed in a microwave dish containing the pre-heated antigen retrieval solution, and were microwaved three times for 5 minutes each. The slides were allowed to cool for 30 minutes and then were rinsed with H_2O and equilibrated in TBS.

2.2.2 Immunohisto/cytochemistry studies.

Slides were equilibrated in TBS for 5 minutes. The slides were blocked with an appropriate serum in which the secondary antibody had been raised. Primary antibody at a concentration specified in the table 2.2 was applied for 2 hours, followed by three 5 minute washes with TBST. Secondary antibody was put on for 30 minutes then washed three times for 5 minutes each with TBST and then visualised using the ABC kit. For immunofluorescence a secondary antibody

conjugated to an Alexa™ dye was used as described below. A negative control was carried out by using TBS instead of the primary antibody.

2.2.2.1 ABC vector red.

ABC was prepared by incubation 5ml of PBA with one drop of reagent A (avidin) and with one drop of reagent B (biotin). After the incubation with the secondary antibody 2 drops of ABC-AP reagent were incubated for 30 minutes. The slides were washed three times for 5 min. each with TBST. Two drops of the vector red stain (2.5ml 100mm Tris.HCl (pH 8.2) + 1 drop A + 1 drop B + 1 drop AP + 25 µl levamisole) were put on the slide and incubated for about 10-20 minutes until colour developed. The slides was washed with water and counterstained with haematoxylin. The slides were mounted using DAKO aqueous mounting medium.

2.2.2.2 Alexa™ dyes

After the primary antibodies the slides were washed three times of 5 minutes each with TBST. An Alexa™ conjugated secondary antibody (diluted 1:200 in serum) was put on the slide and incubated for 3 minutes. The slides were then washed three times for 5 minutes each and mounted using DAKO fluorescent mounting medium. The slides were then visualised using either a ultra-violet (UV) microscope or confocal microscope.

Antibody against	Concentration	Supplier	Catalogue number
CC10	1/1000	Gift[1]	
p21	1/10	DAKO	M7207
p53	1/100	Vector Lab	NCL-p53-CM5p
PCNA	1/5000	SIGMA	P8825
EGFR	1/10	DAKO	M0886
cytokeratin 8	1/10	ICN	10526
cytokeratin 18	1/10	ICN	10500
cytokeratin 19	1/10	ICN	11417
α_5 – integrin	1/10	Gift[2]	
α_v – integrin	1/10	Gift[2]	
α_6 – integrin	1/10	Gift[2]	
β_1 – integrin	1/10	Gift[2]	
β_3 – integrin	1/10	Gift[2]	
β_4 – integrin	1/100	CHEMICON	AB1922
p27	1/200	SIGMA	P2092

Gift[1] - Prof. Gurmukh Singh, Dept. Veterans Affairs Medical Centre, Pittsburgh, Pennsylvania
Gift[2] - Prof. Hideo Yagita, Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan

Table 2.2 - Details of the primary antibody used for immuno-cyto/histochemistry and their relative concentrations.

2.2.3 BromodeoxyUridine (BrdU) incorporation.

Cultured Clara cells were exposed to medium containing 10μM BrdU for 6 hours, after which they were fixed using 80% ethanol at 4°C overnight. After fixation cells were rinsed in PBS for 10 minutes and incubated in 5M HCl for 45 minutes at room temperature. Cells were then washed four times in PBS for 5 minutes each and incubated in 1% H_2O_2 for 10 minutes. Cells were washed twice in PBS for 5 minutes and incubated in blocking solution (PBS + 20% rabbit serum + 0.05% Tween) for 10 minutes. The slides was drained and incubated in rat anti-BrdU antibody (Boehringer Mannheim) diluted 1:10 in blocking solution for 60 minutes. The slide was then washed three times in PBS for 5 minutes each, and DAB solution (100 μl DAB + 4.8ml 0.05M Tris + 100 μl 1% H_2O_2) was added for 5 minutes or until the colour developed. The slides was then rinsed with water and counterstained with haematoxylin, time according to strength of solution. The slide was mounted using DAKO aqueous mounting medium. A negative control was carried out by using TBS instead of the primary antibody.

2.2.4 Lectin studies.

Cytospun cells and plated cells were fixed at appropriate time points in 4% buffered formalin (pH 7.4) for 30 seconds at room temperature. Biotinylated lectins were diluted to a final concentration of 20 μg/ml in TBS, pH 7.4, which contained 10 mM $CaCl_2$, 0.2 mM $MgCl_2$, and 1 mM $MnCl_2$. All incubations were carried out at room temperature. Cells were incubated with lectin for 20 min., rinsed with TBS and incubated for 30 min. with ABC-AP. Binding was visualised using Vector Red. As controls for specificity, lectins were pre-incubated overnight at 4°C with appropriate hapten sugars before incubation with cells (Table 2.3). Lectin binding was completely blocked or significantly weakened by hapten treatment.

Lectin	Blocking sugar
Bauhinia purpurea (BPA)	N-acetyl glucosamine
Concanavalin A (Con A)	D(+) mannose and D(+) glucose
Helix pomatia (HPA)	N-acetyl galactosamine
Maclura pomifera (MPA)	D(+)-galactose
Wheat germ (WGA)	N-acetyl glucosamine

Table 2.3 - List of the lectins used and their relative blocking sugars. (Zieske and Bernstein, 1982; Dobbs et al., 1985; Honda et al., 1989; Geleff et al., 1986)

2.2.5 Nitrotetrazolium blue assay.

The nitrotetrazolium blue (NBT) solution consisted of 100 µl of NBT stock solution (Boehringer Mannheim) in 10 ml staining buffer (10ml 0.1M Tris buffer, pH 9.5; 0.05M $MgCl_2$, 0.1M NaCl) with 0.1% NADPH (10mg in 10ml) (Boehringer Mannheim). Cells were fixed in formalin as above and incubated with the NBT solution for 10 min. at 37°C. Positive cells stained purple.

2.2.6 Feulgen staining

After the culturing cells were fixed in Bouin's fixative overnight at 4°C, the slides were incubated in denaturing solution (5M HCl) for 45 minutes at room temperature. Slides were washed for about 15 minutes using tap water and then incubated in Schiffs reagent for 1 hour at room temperature and then washed in tap water until pink colour developed. Slides were counterstained in 0.1% Light Green and mounted in Cedarwood oil and stored at 4°C in the dark.

2.2.7 Electron Microscopy

Cells were harvested by trypsinisation and washed by centrifugation and resuspension 3 times in PBS. The pellets were then resuspended gently in a 10% glutaraldehyde/PBS solution (v/v) and stored overnight at 4°C. The cells were then centrifuged again (500 g, 5 minutes) and were postfixed in osmium tetroxide, processed to Araldite, sectioned at 0.25μm, and stained with uranyl acetate. Analysis of the sections was carried out in collaboration with Dr. Tim Smith, University of Leicester, England.

2.2.8 Cell counting

In immunocyto/histochemistry a whole range of variation in the degree of staining was observed. Thus, strongly stained cells, cytoplasm or nuclei (as the case may be) were considered as positive staining, while negatively or weakly stained cells were considered as negative. Experiments and counts were repeated at least three times and standard deviation was calculated using Microsoft Excel. Counts of 500 cells were sufficient to achieve a stable running mean.

2.3 Western Blot

2.3.1 Isolation of total protein extracts.

Freshly isolated cells were centrifuged at 500 g for 5 minutes and supernatant removed. The cells were washed three times with 1ml cold PBS and were then lysed using RIPA buffer containing a cocktail of proteinase inhibitors for 30 minutes at 4°C. The cell lysate was scraped from the culture well using a yellow tip and transferred to a clean, cold microfuge tube. 100 µl of RIPA buffer was used to lyse

about 1 x 10^6 cells. The lysate was then centrifuged at 15,000 g for 10 minutes at 4°C. The supernatant was removed and stored at -80°C.

2.3.2 Isolation of cytoplasmic and nuclear protein extracts.

Freshly isolated cells were centrifuged at 500 g for 5 minutes and supernatant removed. Cells were washed three times with 1ml of cold PBS. 100µl of cytoplasmic lysis buffer was added to about 1 x 10^6 cells, vortexed and incubated on ice for 10 minutes. In the case of cultured cells, the same procedure was followed but the washing with ice cold PBS and lysing with cytoplasmic lysis buffer was carried out in the chamber slides containing the cells. The lysate was centrifuged at 6000 g for 10 min. at 4°C and the cytoplasmic extract (supernatant) was removed and stored at –80°C. 100µl of nuclear lysis buffer was added to the remaining cell extract and incubated on ice for 10 minutes. The lysate was centrifuged at 15,000 g for 10 minute at 4°C. The nuclear extract (supernatant) was removed and stored at –80°C.

2.3.3 Immunoprecipitation

IMMUNOcatcher kit (CytoSignal) was used for immunoprecipitation. The sample was divided into equally sized aliquots and place in a microfuge tube. The volume was adjusted to 180µl with lysis buffer. 10µl of primary antiserum directed against the target protein (Table 2.4) was added and incubated for 1 hour at room temperature. 10µl of the protein A/G resin suspension was added and mixed for 30 minutes at room temperature.

Spin filters were pre-coated by filling them with lysis solution for 5 minutes and centrifuged at 14,000 g for 1 minute. The suspension was transferred to pre-coated

spin filters and the microcentrifuge tube was rinsed with 200µl of lysis solution to dislodge any resin sticking to the wall of the tube. The contents were transferred to the spin filter and centrifuged at 14,000 g for 1 minute. The flow-through was discarded and 0.5ml of lysis solution was added to the spin filter and centrifuged at 14,000 g for 1 minute. This was repeated twice.

The spin filter was placed in a clean collection tube and 40µl of SDS-PAGE solution was added to the spin filter and incubated for 15 minutes at room temperature. Care was taken to completely cover the resin with the solution. The protein was collected by centrifugation at 14,000 g for 1 minute and stored at –80°C.

Antibody against	Final Concentration	Supplier	Catalogue number
p21 (C-terminal)	1/10	Santa Cruz	sc-397
p21 (full length)	1/10	Santa Cruz	sc-6246
PCNA	1/10	Sigma	P8825
cdk2	1/10	Sigma	C5223
cdk4	1/10	Sigma	C8218
cdk6	1/10	Sigma	C4976
Cyclin D3	1/10	Sigma	C7214
Cyclin E	1/10	Sigma	P2092

Table 2.4 - Details of the antibodies used for immunoprecipitation and the concentrations at which they were used.

2.3.4 Western Blotting

Proteins for western blot analysis were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-polyacrylamide gel electrophoresis was carried out on precast gels in an XCell II™ tank (Novex) in accordance with manufacturer's instructions. The appropriate protein sample was boiled for 3 minutes and loaded onto a 14% Tris-Glycine precast gel (Novex). Electrophoresis was carried out in running buffer (Appendix 1) at a constant voltage of 125V for about 2.5 hours or until the bromophenol blue dye front reached the bottom of the gel.

Proteins were then transferred onto Hybond™ECL™ nitrocellulose membrane (Amersham) using a Trans-Blot Semi-Dry electrophoresis transfer cell (BioRad) in accordance to manufacturer's instructions. The transfer was carried out in Towbin transfer buffer (Appendix 1) at constant voltage of 15 volts for about 30 minutes.

The nitrocellulose membrane was then removed and washed twice for 10 minutes each in TBST. The membrane was incubated in 10% blocking solution in TBST for 1 hour at room temperature, to prevent non-specific binding. All incubations steps from now on, including the blocking step, were carried out on a revolving apparatus to ensure even exposure of the membrane to all reagents. After blocking, the membrane was briefly rinsed twice with TBST and then washed once for 15 minutes followed by two washes for 5 minutes each. The membrane was incubated in 1/200 diluted primary antibody (Table 2.5) overnight at 4°C. The membrane was then washed (as described above) and incubated in 1/2000 diluted HRP secondary antibody (Table 2.6) for 1 hour. Membrane was then washed once for 15 minutes and then 4 times for 5 minutes each with TBST.

Chemiluminescence detection was carried out using ECL™ Western Blotting detection reagents (Amersham Pharmacia Biotech). The blot was exposed to ECM™ film (Amersham Pharmacia Biotech) and was developed in a

Hyperprocessor developer (Amersham Pharmacia Biotech) in accordance with manufacturer's instructions.

Antibody against	Dilution	Supplier	Catalogue number
p21 (C-terminal)	1/200	Santa Cruz	sc-397
p21 (full length)	1/200	Santa Cruz	sc-6246

Table 2.5 - Details of the primary antibodies used for western blotting and their concentrations

Antibody against	Dilution	Supplier	Catalogue number
anti-rabbit IgG-HRP	1/2000	Santa Cruz	sc-2004
anti-mouse IgG-HRP	1/2000	Santa Cruz	sc-2005

Table 2.6 - Details of the secondary antibodies used for western blotting and their concentrations

2.4 Genotyping of transgenic animals.

2.4.1 Transgenic animals

p21 knockout mice were used in this thesis. p21 knockout mice were obtained from Prof. Philip Leder, Harvard Medical School, Boston, Massachusetts, USA (Deng et al., 1995) Before using the animals the mice were genotyped. DNA was extracted from tail tips and a PCR reaction was carried out.

2.4.2 PCR Reaction.

PCR strategies were accomplished using RedTaq™ DNA polymerase (Sigma) and accompanying reaction buffer. Magnesium chloride (11mM) and potassium chloride (500mM) were included in RedTaq™ buffer at 10x stock concentrations. Pharmacia Biotech supplied all deoxyribonucleoside-triphosphates (dNTPs). PCR reactions were accomplished in a thermocycler (Hybaid) and the PCR products were analysed on 2% agarose gel. Conditions for PCR are summarised in Table 2.6 and 2.7

Genotype	Primer sequences	
p21 wt	Forward (exon 2)	5'TTCTTGTGTTTCAGCCACAG3'
	Reverse (exon 3)	5'GCAGCGTATATCAGGAGACG3'
p21 ko	Forward (neo)	5'CATCGCCTTCTATCGCCTTC3'
	Reverse (exon 3)	5'GCAGCGTATATCAGGAGACG3'

Table 2.7 - PCR primers used to genotype the wt and p21 ko mice as described in (Deng et al., 1995).

Genotype	Melting	Annealing	Extension	No of cycles
p21 wt	94°C, 60 sec	58°C, 60 sec	72°C, 60 sec	30
p21 ko	94°C, 60 sec	58°C, 60 sec	72°C, 60 sec	30

Table 2.8 - PCR thermal profile used to genotype the wt and p21 ko mice as described in (Deng et al., 1995).

2.4.3 Detection of PCR products.

A 2% agarose gel was prepared in TAE buffer and melted in a microwave oven. The agarose solution was allowed to cool to about 50°C and 0.003% of a 10mg/ml ethidium bromide solution was added to the gel. The agarose solution was then poured in a horizontal Submarine Agarose Unit and combs were placed in the gel to form wells. The gel was allowed to set for about 30 minutes after which the combs were carefully removed and the gel placed in the electrophoresis tank in TAE buffer. PCR products were prepared by adding 10 µl of samples to 2 µl of 6 x loading buffer in a microtitration plate. 10 – 20 µl of the samples were run on the gel at 100V for about 1 hour. The gel was removed from the apparatus and visualised on a UV trans-illuminator.

2.5 Statistical Analysis.

Statistical analysis was carried out using Microsoft Minitab software. The general linear model test (ANOVA) with Bonferoni corrections for multiple tests, was used to find out significant changes in cell behaviour upon cell-matrix disruption and to find out differences in Clara cells from wt and p21 ko mice. Experiments and counts were repeated at least three times. For all tests a p value less than 0.05 was considered significant.

Chapter 3 - Regulation of cell cycle control in mouse models of tuberculosis, asthma and bleomycin-induced fibrosis.

3.1 Introduction.

About 20% of the population worldwide die of lung related diseases, 17% of which are due to infections. Asthma and tuberculosis are common diseases in humans. TB is responsible for approximately 3 million deaths per year worldwide, pulmonary fibrosis affect about 100,000 people in the United States, while asthma is to blame for 200,000 deaths per year worldwide.

Various animal models have been used to study the pathogenesis of lung disease but to date few studies have looked at changed in expression of cell cycle control genes in these conditions. The p53 pathway was found to be involved upon exposure of lung epithelial cells to either hyperoxia (O'Reilly et. al, 1998; Barazzone et al., 1998; McGrath 1998), or to a number of DNA damaging agents and carcinogens (Fujishita et al., 1995; Guinee et al., 1996; Gadbois and Lehnert, 1997; Corroyer et al., 1996). The expression of p16 (Sabourin et al., 1998), p21 (Guinee et al., 1996, McGrath, 1998), Rb (Sabourin et al., 1998; Fujishita et al., 1995), c-jun (Dolan et al., 1994; Haase et al., 1997), c-Fos (Haase et al., 1997), TNF-α (Lee and Rannels, 1998; Yao et al., 1998) , TGF-β (Lee et al., 1998), BAX (Guinee et al., 1997) and Bcl-2 (Guinee et al., 1997) were reported to be influenced by a number of DNA damaging agents and pollutants but the exact mechanism is still unclear.

The aim of this chapter is to study the variation in the expression of p21, p53, p27 and PCNA in three common human diseases namely *Mycobacterium tuberculosis* infections, OVA sensitised lungs and Bleomycin-induced fibrosis using animal mouse models and try to find out what is happening in terms of cell cycle control in

each disease. Studying the cell cycle control in these diseases may help in the understanding the cellular and molecular pathology of the diseases and hopefully would lead to better and more effective diagnosis and treatment.

3.2 Tuberculosis

Mycobacterium tuberculosis is responsible for approximately 3 million deaths per year worldwide (Bloom and Murray, 1992). The bacterium is mainly transmitted through the respiratory route and causes tuberculosis infection in 10-15% of infected persons (Caruso et al., 1999). *M. tuberculosis* replicates inside the host macrophages and these activated macrophages are essential to limit the degree of the infection. Macrophages are usually activated by interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), *c-fos* or bacterial products such as lipopolysaccharide (LPS) and lipoarabinomanna to produce reactive nitrogen intermediates (RNI) which are involved in killing intracellular mycobacteria. RNI are required *in vivo* and *in vitro* to control *M. tuberculosis* infections (Roach et al., 1993; MacMicking et al., 1997; Flynn et al., 1998; Barnes et al., 1991). Superoxide dismutase catalyses the conversion of superoxide anion to hydrogen peroxide and contributes to the virulence of intracellular pathogens. Pathogenic mycobacteria produce 93 times more superoxide dismutase as compared with non-pathogenic mycobacteria (Edwards et al., 2001). It is thought that superoxide dismutase contributes to the pathogenesis of tuberculosis by preventing the early elimination of *M. tuberculosis* by innate immune responses, which include the early mononuclear cell infiltration of infected tissues and apoptosis (Tobin, 2002).

3.2.1 Mouse model

Mouse tissue sections of *Mycobacterium tuberculosis* infected lungs were a kind gift of Prof. JoAnne Flynn, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, U.S.A. The method for tuberculosis infection is described in (Caruso

et al., 1999). In brief, specific pathogen-free homozygous C57BL/6 mice, 8-10 weeks old (18-25 g in weight) were infected via the aerosol route with about 75 CFU of Erdman strain of *Mycobacterium tuberculosis*. Mice were sacrificed after day 16, week 4 and week 8.

3.2.2 Histopathology

Initially mice developed focal and non-specific chronic inflammatory infiltrates consisting largely of macrophages and some lymphocytes with only occasional plasma cells. At later time points there was an increase in numbers of fibroblasts, with deposition of collagen. Tissue destruction was minimal and only seen at later time points. Occasional granulomas were seen although caseation was rare and giant cells infrequent (Figure 3.1).

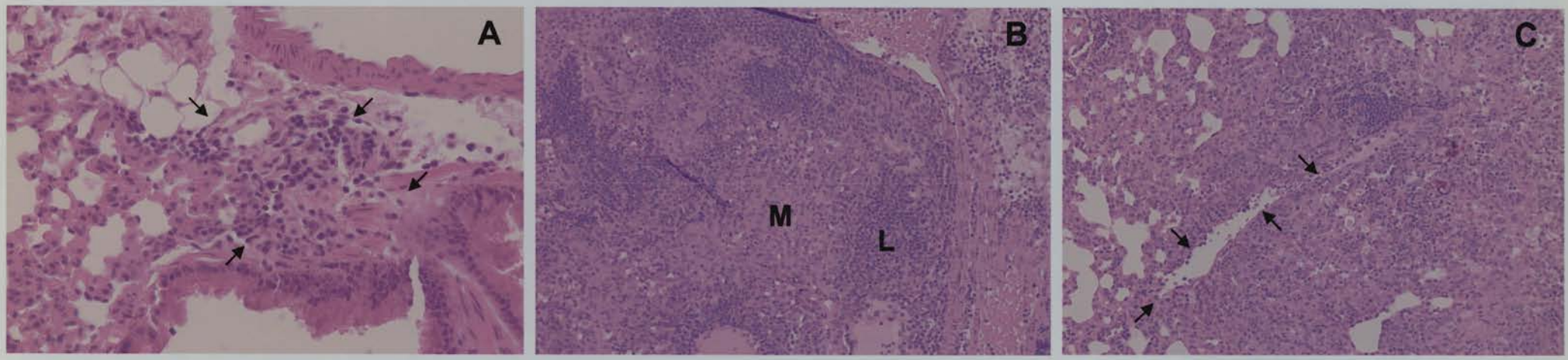


Figure 3.1 – Haematoxylin and eosin lung tissue sections of *Mycobacterium tuberculosis* infections

A Infection at day 16, formalin fixed sections. Peri-bronchiole morphology and lymphocytes. Ill defined granuloma (arrows). Magnification x 100.

B Infection at week 4, formalin fixed sections. Forid confluent inflammation with obliteration but no caseation. Lymphocyte (L) rich, and macrophages (M) rich areas are marked. Magnification x 100

C Infection at week 8, formalin fixed sections. Chronic inflammation which is peri-bronchiolar (bronchiolar wall marked with arrows) and thickening of the alveolar walls. Some increase in fibrosis but classical granuloma formation and caseation is not seen. Magnification x 100.

3.2.3 Immunohistochemistry

Although p21, p27, p53 and PCNA were all present in both bronchiolar and alveolar regions in the lungs of *Mycobacterium tuberculosis* infected lungs at day 16, week 4 and week 8, no difference in the degree and intensity of staining could be seen (Figure 3.3 A, B, C, D, E, F, G, H, I, J, K and L). Although PCNA staining was infrequent in both the bronchiolar and alveolar regions in the disease, PCNA positive lymphocytes were present (Figure 3.2 G, H and I).

Figure 3.2 - Immunohistochemistry of *Mycobacterium tuberculosis* infected lung tissue sections.

A, B and C - p21 immunohistochemistry using Alexa™ 546 conjugated secondary antibody, of formalin fixed lung tissue sections infected at day 16, week 4 and week 8 respectively. Weak cytoplasmic p21 staining in bronchiolar epithelium but no difference over time. Absence of p21 nuclear staining in the bronchiolar epithelium. Magnification x100.

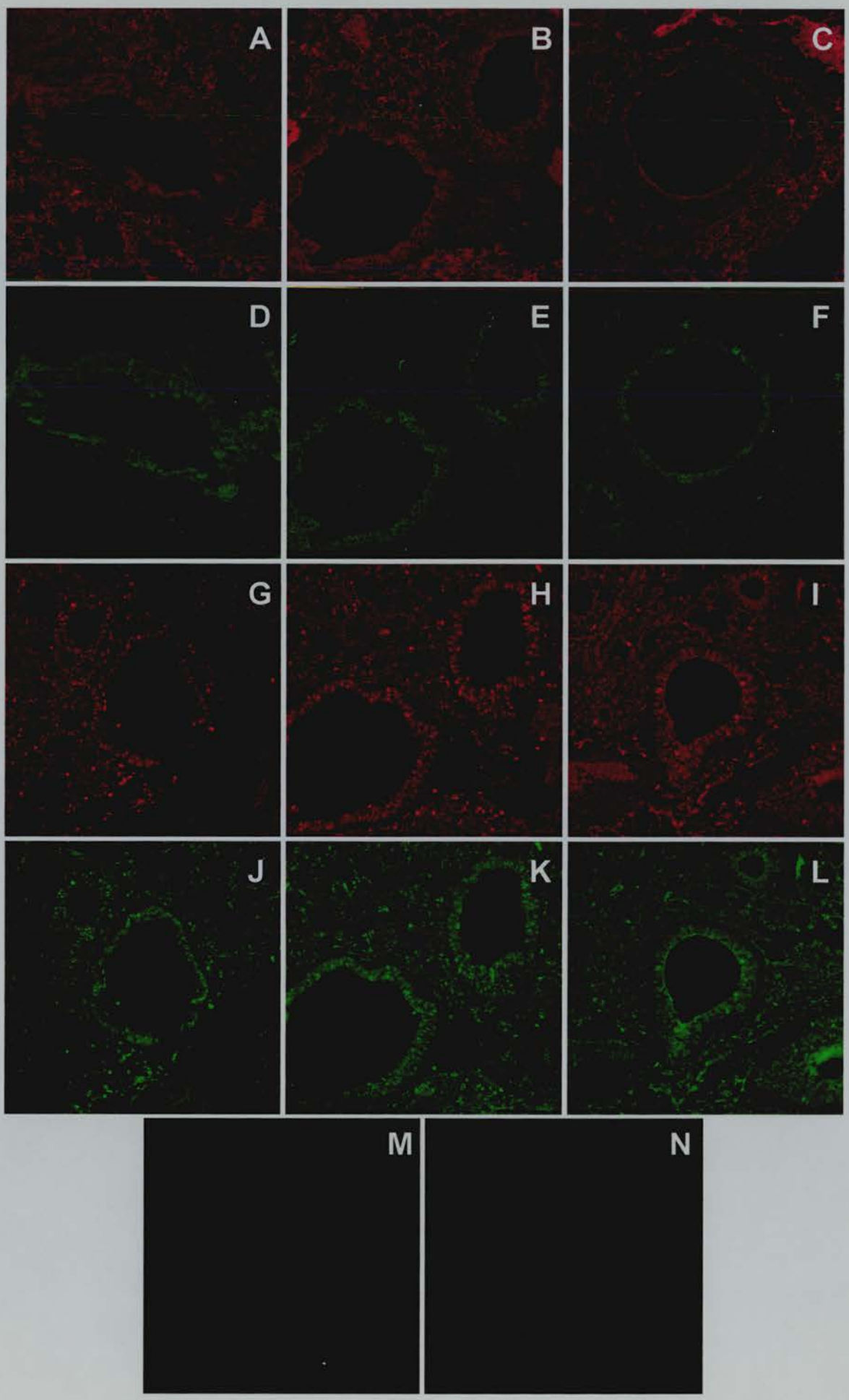
D, E and F - p53 immunohistochemistry using Alexa™ 488 conjugated secondary antibody, of formalin fixed lung tissue sections infected at day 16, week 4 and week 8 respectively. Absence of p53 nuclear staining in both the bronchiolar and alveolar regions. Magnification x100

G, H and I - PCNA immunohistochemistry using Alexa™ 546 conjugated secondary antibody, of formalin fixed lung tissue sections infected at day 16, week 4 and week 8 respectively. Overall cell sporadic nuclear staining in alveolar and bronchiolar epithelium but only increase in inflammatory infiltrates. Magnification x 100.

J, K and L - p27 immunohistochemistry using Alexa™ 488 conjugated secondary antibody, of formalin fixed lung tissue sections infected at day 16, week 4 and week 8 respectively. Overall cell sporadic nuclear staining in alveolar and bronchiolar epithelium. Magnification x 100.

M - Negative control. Primary antibody was omitted whilst using Alexa™ 546 conjugated secondary antibody. No staining. Magnification x 100.

N - Negative control. Primary antibody was omitted whilst using Alexa™ 488 conjugated secondary antibody. No staining. Magnification x 100.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3.3 Ova sensitised lung (Asthma)

Airway inflammation is associated with asthma and is more pronounced with increasing asthma severity (Tobin, 2001; Bousquet et al., 2000). Apoptosis, a dynamic process involved in repair and tissue remodelling tends to limit inflammatory tissue injury and promote resolution rather than progression of inflammation (Druilhe et al., 1998). A significantly higher number of non-apoptotic airway eosinophils and macrophages in subjects with asthma compared with patients with chronic bronchitis has been found. This suggests that these cells can survive longer in the airways of asthmatic subjects (Vignola et al., 2001; Tobin, 2001; Ellia, 2000; Druilhe et al., 1998; Bousquet et al., 2000). The mechanism leading to persistent accumulation of inflammatory cells is not fully understood. It is also unclear whether asthmatics have a primary genetic deregulation of apoptosis pathways or if the reduced apoptosis is a secondary phenomenon in the context of a variety of topically released pro-inflammatory and pro-survival cytokines (Tobin, 2001; Ellia, 2000; Bousquet et al., 2000).

Many different pathways are involved in apoptosis, the main ones being signalling through death receptors such as FAS, TNFR1, the p53 pathway, the Bcl-2 family and the caspases (Wyllie et al., 1994; Wyllie et al., 1998; Wyllie, 1987; Haake and Polakowska, 1993; Guo and Hay, 1999; Gervais et al., 2000; Fleck et al., 1998; Dowen, 1993; Clarke et al., 1995; Bulavin et al., 1999; Brutsche et al., 2001; Druilhe et al., 1998; Bellamy et al., 1995). These complex pathways can lead to pro- and anti-apoptotic effects. Thus a reduction in apoptosis can be due to reduced in the expression of pro-apoptotic factors or increased in the expression of pro-survival products.

Clara cell 10-kDa protein (CC10) (also known as CC16) the predominant product from non-ciliated cells in the epithelial lining, has been shown to have immunomodulatory and anti-inflammatory activity and play a role in controlling airway inflammation (Szabo et al., 1998; Singh and Katyal, 1997; Sagal and Nieto,

1998; Asabe et al., 1998; Hermans et al., 1998b; Hermans et al., 1998a; Broeckaert and Bernard, 2000). CC10 was found to be lower is asthmatic non-smokers patient compared to healthy non-smokers. (Shijubo et al., 1999a; Shijubo et al., 1999b; Laing et al., 2000). A decrease in CC10-positive cells, mainly Clara cells was found in the epithelial lining of bronchioles in smokers who had a normal pulmonary function (Shijubo et al., 1999b; Shijubo et al., 1999a). The decrease in the CC10 production may reflect the remodelling of small airways in asthma (Shijubo et al., 1999a).

The CC16 gene has been screened for mutations and a polymorphism (A38G) was identified and associated with an increased risk of asthma (Laing et al., 2000; Laing et al., 1998b; Laing et al., 1998a).

3.3.1 Mouse model

Mouse tissue sections of OVA sensitised and saline treated lungs were a kind gift of Prof. Paul Foster, John Curtin School of Medical Research, Canberra, Australia. The method for OVA sensitisation and saline treatment of lungs is described in (Xiong et al., 1999; Mould et al., 2000). Briefly, mice 8-10 weeks of age were sensitised by two intraperitoneal injections 50μg OVA in 1mg alhydrogel given on day 0 and 12. Non-sensitised controls received 1mg of alhydrogel in normal saline. On day 24, sensitised mice were exposed to aerosolised OVA (10mg/ml in normal saline) for 30 minute period at intervals of 30 minutes, and the process was repeated every second day for 6 days. Non-sensitised mice received aerosolised saline only. Mice were sacrificed on day 31, 24 hours after the last aerosol.

3.3.2 Histopathology

Inflammatory infiltrates were predominantly peri-bronchiolar. Large numbers of lymphocytes, macrophages, plasma cells, neutrophils were present and a small

number of eosinophils and increased mast cells. The inflammatory infiltrate expands the peri-vascular and peri-bronchiolar connective tissue but there is not definite fibrosis (Figure 3.3).

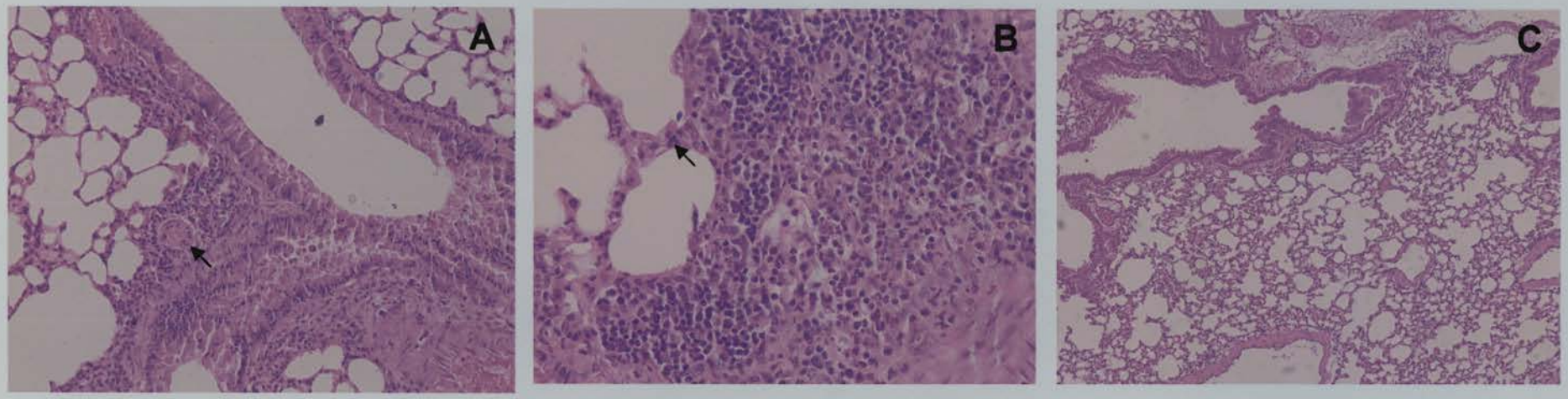


Figure 3.3 – Haematoxylin and eosin staining of OVA sensitised and saline sensitised mouse lung tissue sections.

A OVA sensitised lung tissue section at day 31, formalin fixed. Patchy peri-bronchiolar inflammation and vascular congestion (arrow). Magnification x 200

B OVA sensitised lung tissue section at day 31, formalin fixed. Mixed peri-bronchiolar infiltrates contains both chronic inflammatory cells (lymphocytes and plasma cells) and some granulocytes including eosinophils. Some alveolar wall thickening can be seen (arrow). Magnification x 400.

C Saline sensitised, lung tissue section at day 31, formalin fixed. Mild patchy oedema and inflammation but overall architecture is normal. Magnification x 100.

3.3.3 Immunohistochemistry

There was no difference in the degree and intensity of staining of p27 and PCNA from the OVA-sensitised lung sections compared to the saline-sensitised lung tissue section (Figure 3.4 C, D, E, F, G and H). There was a change in the p53 and p21 staining in the bronchiolar region. In the negative control (non-disease, saline-sensitised lung) p53 and p21 are present in the bronchiolar and alveolar regions. In the OVA-sensitised asthmatic/hyperreactive lung p53 and p21 was not detected in the bronchiolar region but still present in the alveolar regions (Figure 3.4 A and B). Thus there seem to be a decrease in the p53 and p21 expression in the bronchiolar region of the OVA-sensitised asthmatic/hyperreactive lung.

Figure 3.4 - Immunohistochemistry of OVA sensitised and saline sensitised mouse lung tissue sections.

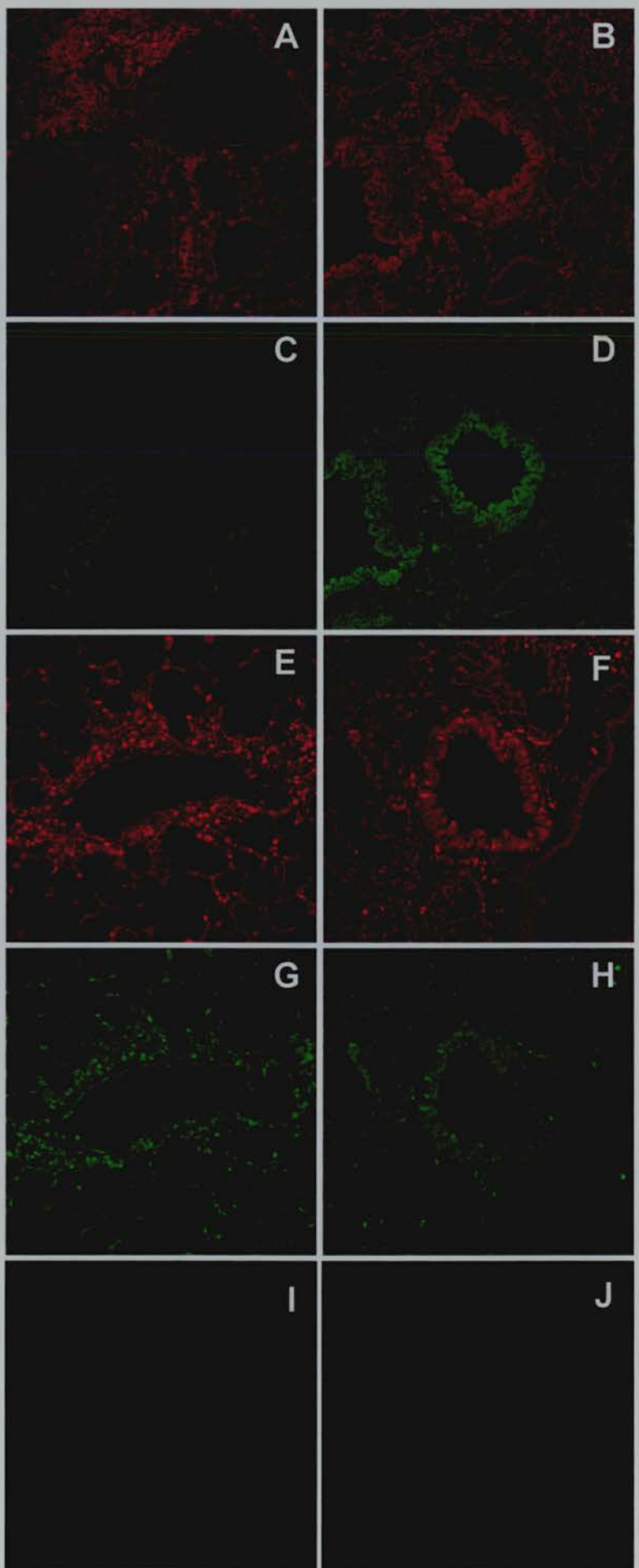
A and B - p21 immunohistochemistry using Alexa™ 546 conjugated secondary antibody of OVA sensitised and saline sensitised formalin fixed lung tissue sections respectively. No positive nuclear staining in the bronchiolar region but some staining in the alveolar regions. There is a decrease in the cytoplasmic staining in the OVA sensitised lungs (A) compared to the saline sensitised (B). Magnification x 100

C and D - p53 immunohistochemistry using Alexa™ 488 conjugated secondary antibody of OVA sensitised and saline sensitised formalin fixed lung tissue sections respectively. No positive nuclear staining in the alveolar and bronchiolar epithelial regions. The cytoplasmic expression of p53 decreases in the OVA sensitised lungs (C) when compared to the saline sensitised (D). Magnification x 100

E and F - PCNA immunohistochemistry using Alexa™ 546 conjugated secondary antibody of OVA sensitised and saline sensitised formalin fixed lung tissue sections respectively. Nuclear and cytoplasmic staining present in both alveolar and bronchiolar epithelial regions. No difference in PCNA expression between the OVA sensitised lungs (E) and saline sensitised (F). Magnification x 100

G and H - p27 immunohistochemistry using Alexa™ 488 conjugated secondary antibody of OVA sensitised and saline sensitised formalin fixed lung tissue sections respectively. Nuclear and cytoplasmic staining present in both alveolar and bronchiolar epithelial regions. No difference in p27 expression between the OVA sensitised lungs (G) and saline sensitised (H). Magnification x 100

I and J - Negative controls. Primary antibody was omitted whilst using Alexa™ 488 and 546 conjugated secondary antibody respectively. No staining. Magnification x 100.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3.4 Bleomycin induced fibrosis

Pulmonary interstitial fibrosis is defined as thickening and stiffening of the lining of the air sacs (alveoli) of the lungs causing progressive breathlessness (Crystal et al., 1976; Crystal et al., 1978; Crouch, 1990; Cantin et al., 1987).

The pathology of Idiopathic Pulmonary Fibrosis (IPF) also known as Cryptogenic Fibrosing Alveolitis (CFA) shows an initial alveolitis with persistent accumulation of inflammatory cells including neutrophils, alveolar macrophages and lymphocytes in the lower respiratory tract. This is followed by parenchymal cell injury including extensive destruction of type I alveolar epithelial cells and re-population of the epithelial cell surface by proliferating type II alveolar epithelial cells, resulting in alterations in lung extracellular matrix, fibroblast proliferation and enhanced collagen synthesis with the accumulation of excess connective tissue in the lung (Crystal et al., 1976; Crystal et al., 1978; Crouch, 1990; Christensen et al., 1999; Cantin et al., 1987).

3.4.1 Mouse model

Mouse tissue sections of bleomycin-induced fibrosis and saline treated (negative controls) were a kind gift of Prof. Thomas Strandjord, Department of Pediatrics, Seattle, USA. The method for bleomycin-induction is described in (Hormuzudi et al., 1999). In brief, specific pathogen-free homozygous C57BL/6 mice, 8-10 weeks old (18-25 g in weight) were treated 3.5mU/g bleomycin sulfate in 2.33µl/g sterile saline given via transtracheal puncture, under intraperitoneal Avertin anesthesia. Lungs were harvested at 14 days after instillation after overdose of intraperitoneal Avertin anesthesia and pulmonary arteries perfused with PBS. The left lung was fixed by intracheal instillation of 4% paraformaldehyde in PBS at 30cm H_2O pressure for 2 hours, then fixed overnight in 4% paraformaldehyde in PBS at 4°C and

embedded in paraffin. For the bleomycin negative control, the mice were treated with transtracheal sterile saline alone, 2.33μl/g.

3.4.2 Histopathology

The injury following intratracheal bleomycin-injury tends to be patchy, which probably reflects where bleomycin droplets go, and variable in intensity. Later the inflammatory response was fairly intense and widespread (Figure 3.5 A and B). The saline-treated lung appears completely normal (Figure 3.5 C).

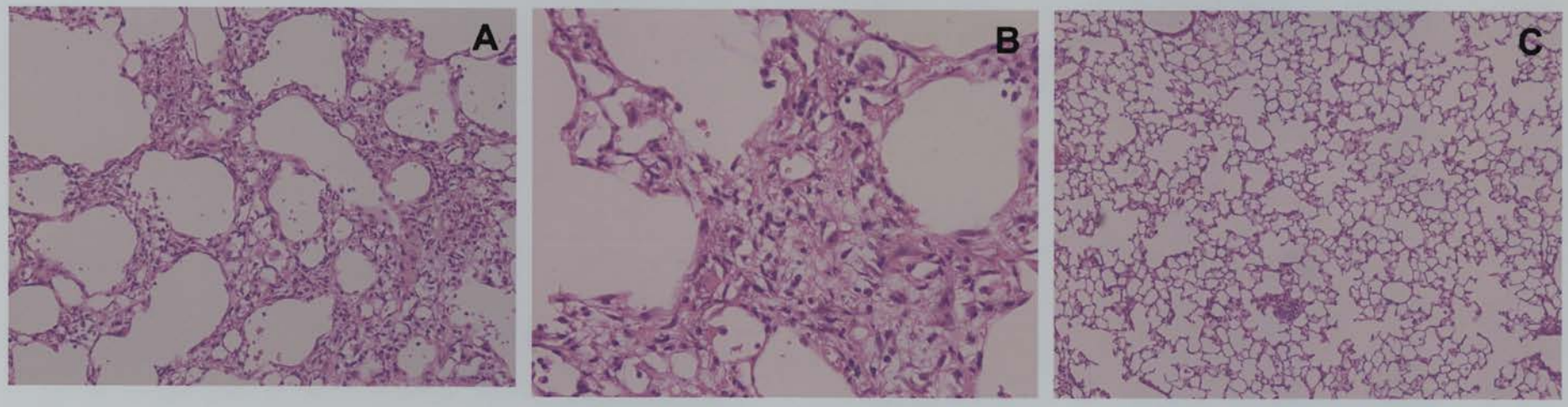


Figure 3.5 Haematoxylin and eosin staining of bleomycin-treated and saline treated mouse lung tissue sections.

A and B - Bleomycin induced fibrosis at day 14, formalin fixed sections. Normal focal area of alveolar wall. Thickening with an increase in cells in the interstitium most of which have spindle morphology typical of fibroblasts. Admixed within this are inflammatory cell including macrophages.

A Magnification x200

B Magnification x400

C Negative control (Saline-induced) at day 14, formalin fixed sections. No significant abnormalities in the lung architecture. Magnification x 100

3.4.3 Immunohistochemistry

There was no difference in the degree and intensity of staining of p53 and PCNA from the bleomycin-induced fibrosis lung sections compared to the saline-induced non-fibrotic lung tissue section (Figure 3.6 C, D, E and F). The degree of staining of p27 seems to be the same in fibrotic tissue compared to the non-fibrotic but the intensity seems to be higher (Figure 3.6 G and H). It is quite difficult to explain why, since this could be an experimental artifact or that that actually more cells are expressing or stabilising more p27 following the bleomycin-injury/disease. Although no nuclear p21 expression was found, the level of cytoplasmic p21 expression (both degree and intensity of staining) is higher the bronchiolar and alveolar regions in the fibrotic lung compared to the non-fibrotic lung tissue sections (Figure 3.6 A and B).

Figure 3.6 - Immunohistochemistry of bleomycin-treated and saline-treated mouse tissue sections.

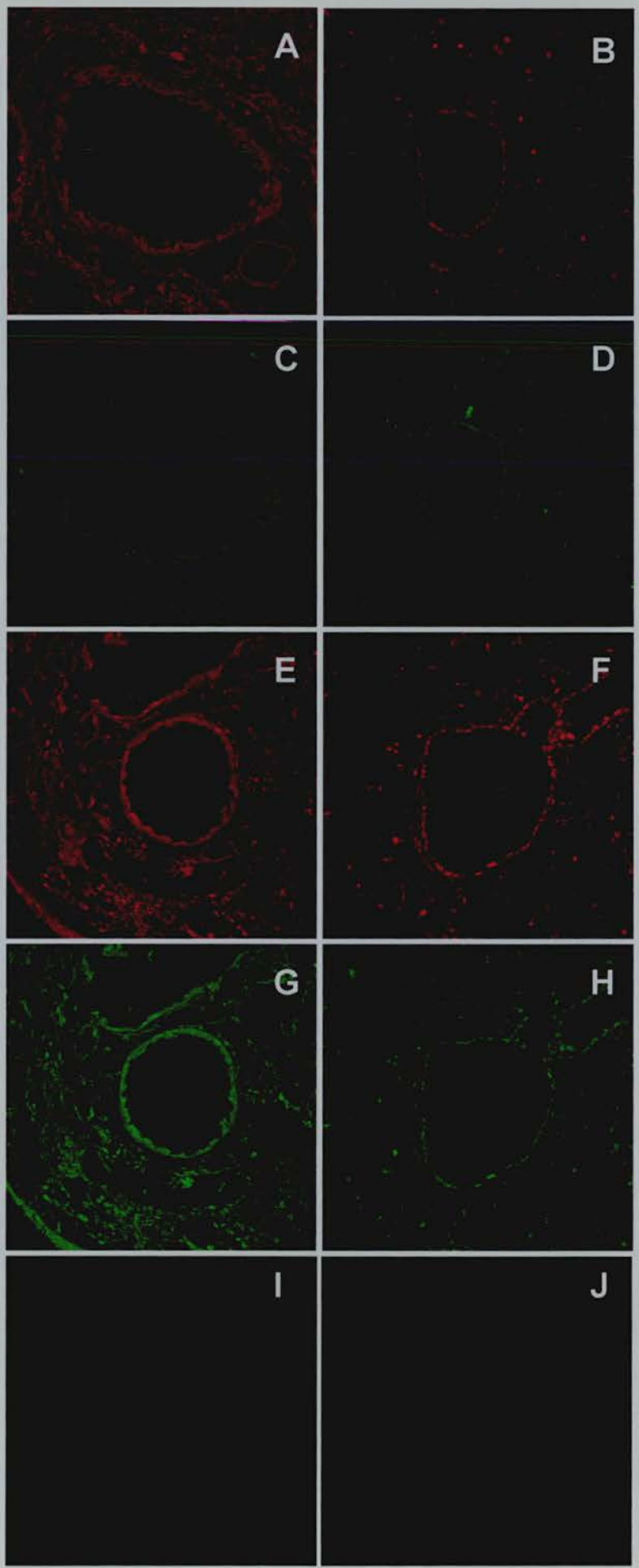
A and B - p21 immunohistochemistry using Alexa™ 546 conjugated secondary antibody in bleomycin treated and saline treated formalin fixed lung tissue sections respectively. Nuclear and cytoplasmic staining present in both alveolar and bronchiolar epithelial regions. p21 expression is greater in the fibrotic lung (A) and non-fibrotic lung (B). Magnification x 100.

C and D - p53 immunohistochemistry using Alexa™ 488 conjugated secondary antibody in bleomycin treated and saline treated formalin fixed lung tissue sections respectively. Cytoplasmic staining present in both alveolar and bronchiolar epithelial regions in both fibrotic (C) and non-fibrotic lungs (D). Magnification x 100.

E and F - PCNA immunohistochemistry using Alexa™ 546 conjugated secondary antibody in bleomycin treated and saline treated formalin fixed lung tissue sections respectively. Nuclear and cytoplasmic staining present in both alveolar and bronchiolar epithelial regions. No difference between fibrotic (E) and non-fibrotic (F) lungs. Magnification x 100.

G and H - p27 immunohistochemistry using Alexa™ 488 conjugated secondary antibody of bleomycin treated and saline treated formalin fixed lung tissue sections respectively. Nuclear and cytoplasmic staining present in both alveolar and epithelial regions. An increase p27 expression in fibrotic (G) compared to non-fibrotic lungs (H). Magnification x 100

I and J - Negative controls. Primary antibody was omitted whilst using Alexa™ 488 and 546 conjugated secondary antibody respectively. No staining. Magnification x 100.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3.4.4 Gene expression analysis.

The gene expression studies were carried out in collaboration with Prof. Dean Sheppard's group, University of California, San Francisco, U.S.A. The method is described in (Kaminski et al., 2000). Protein distribution was assessed by immunohistochemistry in Edinburgh and parallel gene array studies in USA. Briefly, total RNA was isolated from bleomycin-treated mice and saline treated mice 7 or 14 days after treatment. cRNA was prepared and hybridised to GENECHIP array. The GENECHIP array was then analysed using GENECHIP 3.1 software (Affymetrix).

The gene expression data for p21, p53, PCNA and CC10 is shown in Table 3.1. From the data it can be seen that p53 and PCNA did not change significantly from the fibrotic lungs generated by bleomycin treated lungs at day 7 and at day 14 compared to the negative control, saline treated lung. Two CC10 markers have been used, only one of which gave a significant result in the fibrotic lung at day 7. Thus no significant conclusion can be obtained from the CC10 results.

An interesting result was obtained regarding p21 expression. The levels of p21 expression in fibrotic lung at 14 days were statistically significant higher ($p = 0.05$) when compared to the negative fibrotic lung. This data fits quite well with the results obtained by immunohistochemistry (Figure 3.7), whereby p21 expression was found to be higher in the bleomycin injured lungs at day 14 compared to the negative fibrotic lung. From the immunohistochemistry studies there was no variation in the levels of p53 and PCNA.

Gene	Genbank Acc. No.	RNA levels of normal lungs	RNA levels of fibrotic lungs at day 7	p value (fibrosis at day 7 vs normal lungs)	RNA levels in fibrotic lungs at day 14	p value (fibrosis at day 14 vs normal lungs)
p21	U09507	231	392.7	0.12	435.3	0.05
p53	U59758	30.8	25.0	0.77	66.7	0.31
p53	X01237	187.2	146.7	0.57	162.3	0.74
PCNA	AA146128	20.0	13.3	0.42	20.0	1
PCNA	X57800	342.4	409.7	0.57	392.7	0.68
CC10	AA028748	3119.2	3042.7	0.95	3138.0	0.98
CC10	L24372	2282.8	1292.7	0.01	1789.3	0.21

Table 3.1 - Gene expression analysis on saline treated lung (normal), bleomycin treated (fibrosis day 7) and bleomycin treated (fibrosis day 14) mouse lung.

3.5 Discussion

3.5.1 Bleomycin induced fibrosis.

Bleomycin, a potent cancer chemotherapeutic agent, causes fibrogenic lung disease in rats and mice similar to that seen in human subjects. Bleomycin affects many cellular pathways, but it is believed that the cytotoxic effect is due to its ability to bind and cleave DNA bleomycin (Mori et al., 1989). A number of reports indicate that bleomycin generates reactive oxygen species *in vitro* (Yi et al., 1998; Wu et al., 1998b; Mori et al., 1989; Daly et al., 1998; Daly et al., 1997).

The p53 tumour suppressor protein is a DNA damage-inducible protein (Zaika et al., 1999; Wyllie et al., 1994; Wu et al., 1998a; Vogelstein et al., 2000; Sigal and Rotter, 2000; Sheikh et al., 1997; May and May, 1999; Matlashewshi, 1999; Lane, 1992; Lakin and Jackson, 1999; Kaelin, 1999a; Kaelin, 1999b; Brambilla and Brambilla, 1997; Ashcroft and Vousden, 1999; Albrechtsen et al., 1999). Thus one would expect to find an increase in p53 expression in a bleomycin induced-fibrosis model. In the bleomycin-induced fibrosis mouse model there was no difference in the degree and intensity of staining of p53 in the fibrosis (at day 14) and non-fibrotic tissue sections. In the gene expression analysis no statistically significant changes in the p53 expression was found in fibrotic lungs generated by bleomycin treated lung at day 7 and at day 14 compared to the non-fibrotic lungs. Mishra et al., 2000, found that there was an increase p53 immunostaining in the lung cells of bleomycin induced fibrosis tissue at day 3 and day 7 but a decrease in day 9 and no evidence of p53 expression at day 14.

In this study no differences was found in the immunostaining of PCNA from the fibrotic (day 14) to non-fibrotic tissue. Also the degree of PCNA expression as determined by gene expression analysis was the same in fibrotic lungs (at days 7 and 14) and non-fibrotic lungs. This data was similar to the data by (Mishra et al., 2000).

From immunostaining of p21, the level of p21 staining was found to be higher in fibrotic (day 14) tissue compared to non-fibrotic tissue. The level of p21 expression in fibrotic lungs at day 7 was not statistically higher when compared to the non-fibrotic lungs. At day 14, the expression of 21 was statistically higher ($p = 0.05$) when compared to non-fibrotic lungs. Mishra et al., (2000) found out p21 overexpression at day 5 and day 7 after exposure but most intense p21 nuclear staining in epithelial cells was at day 9.

The level of mRNA levels of p53 and p21 have been studied in bleomycin induced fibrosis lungs at 1 hours, 6 hours, 1, 3, 5, 7 and 14 days by Kuwano et al., (1996 and 2000). Kuwano et al., (2000) found out that lung mRNA levels of p53 and p21 were upregulated at 1 hour to day 7 after bleomycin instillation, while only p21 mRNA, but not p53 mRNA was detectable at 14 days.

From the above data it appears that following bleomycin induction, an increase in p21 induction via p53-dependent pathways may be involved. Since p53 were mainly induced in the early phase, it is likely that p53 may have an important role in the initial phase of the disease, that is when DNA were mainly damaged by bleomycin itself but not at a later stage when DNA was damaged by infiltrating inflammatory cells.

p21 is most probably playing an important role in the p53-induced G1 arrest because it is a potential inhibitor of cyclin-dependent kinase activity (Wu et al., 1998a; Tomoda et al., 1999; Sheikh et al., 1997; Rousseau et al., 1999; Haapajärvi et al., 1999; Cheng et al., 1999). It has been shown previously that serum or individual growth factors such a platelet-derived growth factor, fibroblast growth factor and epidermal growth factor but not insulin are able to induce p21 in quiescent p53-deficient cells as well as normal cells (Michieli et al., 1994). Thus the induction of p21 without up-regulation of p53 mRNA at 14 days after bleomycin-induction may be dependent on growth factors, such as platelet-derived growth factor and fibroblast growth factor.

Apart from direct DNA damage from bleomycin and subsequent cell death, fibrosis could have resulted from inflammatory cell recruitment, fibroblast proliferation or collagen synthesis. Inflammatory cells, including neutrophils and monocytes can release toxic oxygen reactive species that can damage the DNA (Sato and Muramatsu, 1985; Sato et al., 1999).

Recent evidence suggests that cell-ECM interaction may be more important in modulating fibrosis than simply the degree of inflammation (Sheppard, 2001b). Although many of the normal lung development processes are still unknown, it is clear an important part of the network of reciprocal inductive and inhibitory signals involves the mesenchymal cells, pulmonary epithelial cells and components of the ECM (Ebihara et al., 2000).

Several lines of evidence suggest that TGF-β is involved in the regulation of pulmonary fibrosis (Coker et al., 1996; Gauldie et al., 2002). TGF-β1 has been shown to be activated via integrins mainly αvβ6 (Sheppard, 2001a). The binding and activation of latent or inactive TGF-β1 could be a mechanism whereby pulmonary fibrosis and inflammation could be regulated (Munger et al., 1999). It has also been shown that β6 integrin expression is associated with sites of neutrophil influx in lung epithelium (Miller et al., 2001).

Mice that are homozygous for a null mutation of the β6 subunit gene phenotypically exhibit pronounced lymphocyte accumulation in conducting airways, suggesting that the αvβ6 integrin complex may play a role in down-regulating inflammation in the lung (Huang et al., 1996). β6 knockout mice are protected from development of TGF-β1 dependent bleomycin-induced pulmonary fibrosis, yet leukocyte recruitment into the lung after treatment with bleomycin was not inhibited (Munger et al., 1999). This further suggests the important role of αvβ6 during inflammatory events in the airways.

From immunostaining, the degree of p27 staining was found to be the same but the intensity seems to be higher in fibrotic (day 14) tissue compared to non-fibrotic tissue. The increase in the degree of p27 staining could be either due to probable increase in TGF-β or due to changes in cell-cell interactions (Slingerland and Pagano, 2000; Lloyd et al., 1999). It is quite difficult to make any conclusion about p27 since the effects of TGF-β, rapamycin, and contact inhibition on cell proliferation remained unchanged in p27 null mice (Nakayama et al., 1996).

From the gene expression analysis studies, only one CC10 marker gave a significant result in fibrotic lung at day 7, but no significant conclusion can be made since only one of the CC10 was statistically significant. A decrease in the number of Clara cells evaluated by CC10 mRNA expression in the airway epithelial lining was described at 28 days following bleomycin induction (Daly et al., 1997). Daly et al., 1998 suggested that where CC10 mRNA expression was lost and SPB mRNA remained the same thus the cells could be an intermediate cell type that is a precursor to either mature Clara cells or ciliated cells.

3.5.2 OVA-sensitised lungs.

There was no difference in the degree and intensity of staining of p27 and PCNA from the OVA-sensitised lung sections compared to the saline-sensitised lung tissue section. It was previously reported that PCNA was highly increased in the epithelium from steroid-treated asthmatics (Vignola et al., 2001; Druilhe et al., 1998). It was suggested that steroid could facilitate the process of epithelium proliferation and survival (Druilhe et al., 1998).

In the negative control (non-disease, saline-sensitised lung) p53 and p21 are present in the bronchiolar and alveolar regions while in the OVA-sensitised asthmatic/hyperreactive lung p53 and p21 seems not to be present in the bronchiolar region but still present in the alveolar regions. There seems to be a decrease in the p53 and p21 expression in the bronchiolar region of the OVA-sensitised

asthmatic/hyperreactive lung. A reduction in the levels of p53, together with an increased amount of granulocyte-macrophage colony-stimulating factor (GM-CSF) and Bcl-2 has been described in bronchiolar biopsy (Vignola et al., 1999). An increase in the expression of p53-neutralising protein MDM2 and a four-fold reduction of ataxia telangiectasia gene (ATM) had been described (Brutsche et al., 2001).

A reduction in p53 and p21 expression in asthmatic lung could result in the reduced induction of apoptosis via the p53 pathway. A role for reduced apoptosis has been postulated as contributing towards accumulation of inflammatory cells in the lungs of asthmatics (Vignola et al., 1999; Druilhe et al., 1998; Brutsche et al., 2001).

The immunostaining of p27 was similar in OVA-sensitised lungs as compared to saline treated lungs. One of the roles of p27 is to promote apoptosis and this could be another reason for the decrease in apoptosis in asthmatic lungs.

3.5.3 *Mycobacterium tuberculosis* infected lungs.

Although p21, p27, p53 and PCNA are all present in both bronchiolar and alveolar regions in the lungs of *Mycobacterium tuberculosis* infected lung at day 16, week 4 and week 8 no difference in the degree and intensity of staining could be visualised. Although it is known that in *M. tuberculosis* reactive nitrogen intermediates (RNI) (Roach et al., 1993; MacMicking et al., 1997; Flynn et al., 1998; Barnes et al., 1991) and superoxide dismutase (Edwards et al., 2001) are involved in contributing the virulence of intracellular pathogens, these do not seen to damage the DNA and thus the p53 pathways in most probably not involved.

3.5.4 Conclusions.

p21 expression was increased in bleomycin-induced fibrosis, decreased in OVA-sensitised lungs, a model for asthma and while no changes in the expression of p21 were observed in *Mycobacterium tuberculosis* infected lungs. p21 expression was found to increase independent of p53 at 14 days after bleomycin-induction. Thus other factors such as cell-cell interactions, cell-integrin interactions, changes in ECM composition and time scale could determine the expression of p21.

Since p21 is known to be involved in cell proliferation, death and differentiation, it is important to study p21 expression in a progenitor cell type such as Clara cells. p21 was found to be expressed both in the cytoplasm and in the nucleus. From the literature little is known about the functional role of cytoplasmic p21 thus further studies need to be carried out to determine its role in lung injury and diseases.

Chapter 4 – Isolation and Characterisation of Clara cells.

4.1 Introduction

After a lung injury, epithelial cells are damaged and shed leaving the basement membrane 'nude'. The normal response to injury is usually a mass transportation of factors including extracellular matrix (ECM), various growth factors and inflammatory cells that help regenerate the lost epithelial cells (Persson et al., 1996; Erjefält et al., 1997; Erjefält et al., 1996; Erjefält and Persson, 1997; Erjefält et al., 1995; Rennard, 1999). This regeneration is carried out by stretching of the stem cell to cover the exposed area, proliferation and differentiation.

Although a number of publications suggest possible mechanisms of lung repair after an insult or injury, it is still not fully understood. In the mouse, bronchiolar region Clara cells contribute to a secretion to the extracellular lining fluid and are progenitor cells for self replication and for ciliated cells. They contain a variety of cytochrome p450 monooxygenases that have an active role in the metabolism of xenobiotics.

Relatively few studies have been carried out so far to maintain Clara cells in culture (Van Winkle et al., 1996a; Oreffo et al., 1990; Masek and Richards, 1990; Devereux and Fouts, 1980; Devereux, 1984; Belinksy et al., 1995). Thus the aim of this chapter is to:

1. Isolate Clara cells from mouse
2. Culture Clara cells in serum free condition
3. Characterise *in vivo* and *in vitro* Clara cells in terms of proliferation, differentiation, death and cell cycle control regulatory proteins.

4.2 Clara cell isolation and culturing.

Clara cells have been characterised previously (Belinksy et al., 1995; Devereux, 1984; Devereux and Fouts, 1980; Oreffo et al., 1990; Masek and Richards, 1990; Richards et al., 1990; Chinet et al., 1997; McBride et al., 2000). In this study various modifications of original technique were carried out in order to improve purity and obtain healthy cells and to standardise the method. Some of these modifications included the use of plastic and glass slides treated in various ways including electrical treated plastic chamber slides, glass chamber slides treated with fibronectin and collagen IV, glass chamber slides treated with fibronectin, and glass chamber slides treated with collagen IV. The best results were obtained when using glass chamber slides treated with 50 μg/ml fibronectin. The extracellular matrix (ECM) should be further studied to improve the cell attachment to the chamber slides and also to study the effect of the ECM on cell differentiation and proliferation (Sannes et al., 1996; Dunsmore and Rannels, 1996; Sannes et al., 1998)

From one mouse approximately 250,000 cells were isolated most of which are Clara cells as determined by CC10 expression (section 4.3), NBT assay (section 4.4) and electron microscopy (section 4.7). Oreffo et al., (1990) obtained about 550,000 cells from mice containing about 68% Clara cells. Typical Clara cells at time 0 and 120 hours in culture are shown in figure 4.1.

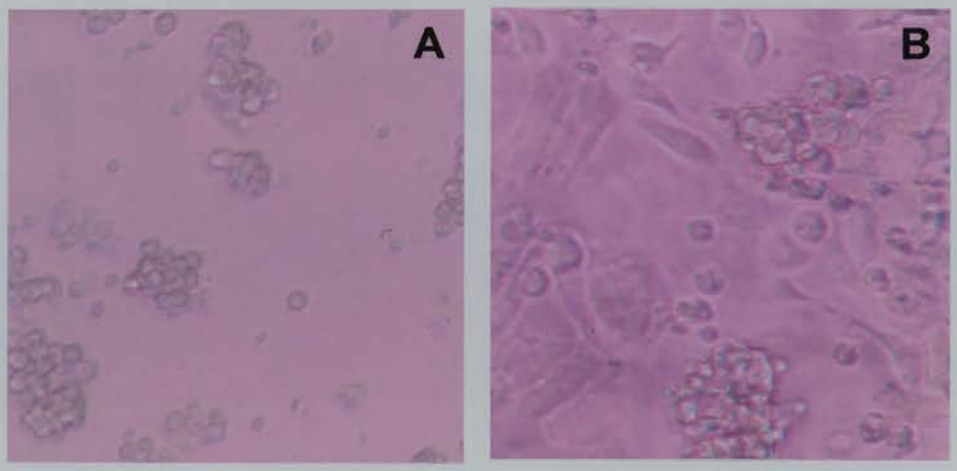

Figure 4.1 - Clara cell culture. Freshly isolated Clara cells at (time 0 hours), most cells are in clumps and are moving around (A). Clara cells at 120 hours in culture, most cells have attached to the ECM and spread. Some cells still remain in clumps (B). Magnification of x100.

4.3 Clara cells 10kDa protein (CC10) expression.

CC10 immunoreactivity was demonstrated in the apical membrane regions of bronchiolar cells in mouse lung tissue (figure 4.2A). Freshly isolated cells (time 0 hours) were strongly positive for CC10 immunoreactivity and remained so at time 72 hours in culture (figure 4.2B). Staining appeared stronger within clumps of cells that had not spread fully compared with larger, flattened cells. At 120 hours in culture, CC10 immunoreactivity was slightly reduced (figure 4.2C).

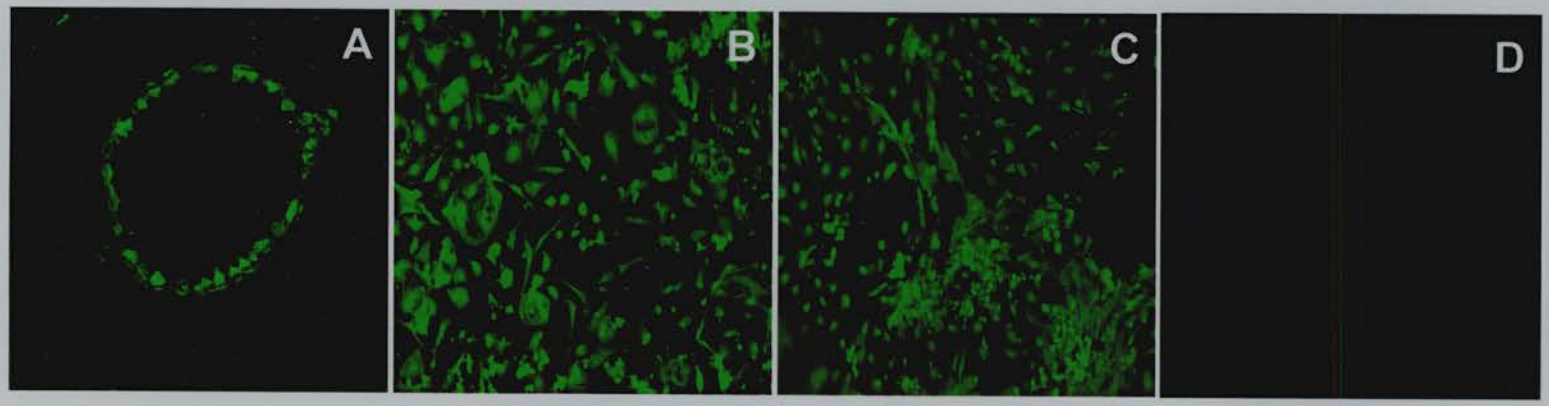

Figure 4.2 - Immunofluorescence of CC10 using Alexa™ 488 conjugated secondary antibody.
(A) CC10 cytoplasmic staining in the apical membrane regions of bronchiolar cells in mouse lung tissue. Strong positive CC10 immunoreactivity in Clara cells cultured at 72 hours (B) and 120 hours (C). (D) is a typical negative control whereby the primary antibody was omitted. Magnification x200

4.4 Nitrotetrazolium blue (NBT) assay.

Functional Clara cells contain high levels of the enzyme NBT reductase (Devereux and Fouts, 1980; Oreffo et al., 1990). This can be detected histochemically using an NBT in which a colourless NBT solution is converted a purple formazan product by NBT (reductase in the presence of NADPH). Brief fixation with formalin prior to staining eliminated the low levels of NBT reductase activity present in other cell types (Oreffo et al., 1990). Following staining, approximately 75% freshly isolated cells were dark purple in colour, indicating that these were functional Clara cells (Figure 4.3A). Most positive cells were present within clumps. The number of NBT-positive cells declined in culture. At day 1 and day 5, approximately 50% and 40% respectively stained purple (Figure 4.3B). Again, most positive cells were present within clumps of cells which had not spread fully, rather than in the areas of flattened, spread cells.

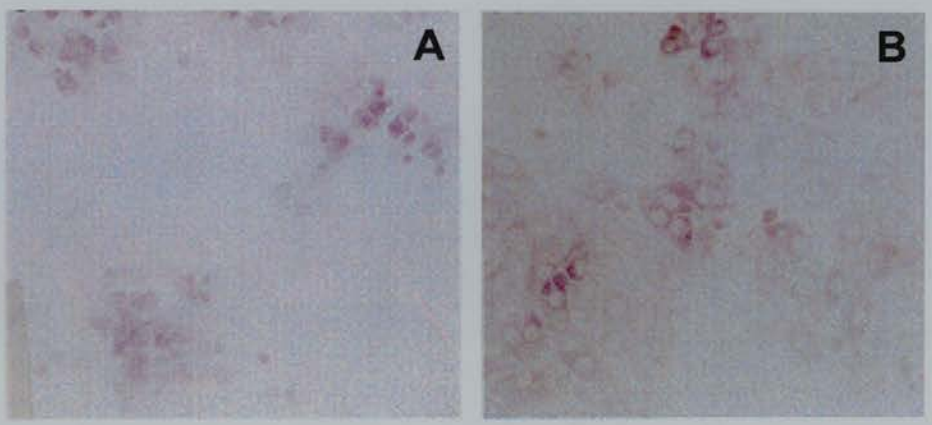

Figure 4.3 - Nitrotetrazolium blue (NBT) assay
A Freshly isolated Clara cells, approximately 75% of cells were NBT positive, magnification x 200
B Cultured Clara cells at day 5, approximately 40% of cells were NBT positive, magnification x 200.

4.5 Lectin characterisation.

Lectins have been used as tools to distinguish between cell types on the basic of their binding to specific carbohydrates groups, usually at the cell surface (Geleff et al., 1986; McBride et al., 2000). These lectin binding sites may be involved in intracellular recognition and binding to yeast, bacteria and other microorganisms to host cells (Sato and Muramatsu, 1985). The specific nature of these interactions means that lectins are potentially useful markers for early epithelial changes in lung diseases as well as markers for *in vitro* differentiation investigations (Joyce-Brady and Brody, 1990; Kasper et al., 1994).

The binding activities of 5 lectins (BPA, *Bauhinia purpurea* - pea; ConA, *Canavalia ensiformis* - jack bean; HPA, *Helix pomatia* - snail; MPA, *Maclura pomifera* - osage orange and WGA, *Triticum vulgarare* - wheat germ, were examined to mouse bronchiolar cells over 5 days in culture (Table 4.1).

In mouse lung tissue sections BPA, MPA, Con A and WGA reacted strongly with the apical membrane of bronchiolar Clara. HPA showed no reactivity. Lectin binding was completely blocked or significantly weakened by control hapten treatment. Figures 4.4 A and 4.4 D illustrated lectin binding of BPA and MPA in mouse tissue sections respectively. WGA and MPA bind strongly to the apical membranes of bronchiolar epithelial cells and also to alveolar epithelial cells. BPA also binds to apical membranes of bronchiolar cells, but more weakly than WGA and MPA. BPA was also found to bind to alveolar cells. ConA was found to bind strongly to all types of cells.

ConA and WGA both showed very strong binding activity to freshly isolated cells, and all cells were stained. Staining remained strong at 24 hours and 120 hours in culture, thus there seem to be no variation during cell culture. BPA showed no reactivity with freshly isolated cells and only few scattered positive cells (less 1%) were present at 24 hours in culture (Figure 4.4 B). However, at 120 hours in culture,

30-40% cells bound BPA (Figure 4.4 C). MPA produced moderately strong positive staining in freshly isolated cells and cultured cells at 24 and 120 hours were all strongly positive (Figures 4.4 E and 4.4 F). HPA showed no reactivity with isolated or cultured cells at any stage.

	BPA	ConA	HPA	MPA	WGA
lung tissue	++	++++	++	+++	+++
cells d0	0	++++	0	++	+++
cells d1	±	++++	0	+++	+++
cells d5	++	++++	0	+++	+++

Key:
- ++++ very strong positive
- +++ strong positive
- ++ moderately positive
- + weak positive
- ± very weak positive
- 0 no stain

Table 4.1 - Variation of lectin expression (BPA, *Bauhinia purpurea* - pea; ConA, *Canavalia ensiformis* - jack bean; HPA, *Helix pomatia* - snail; MPA, *Maclura pomifera* - osage orange and WGA, *Triticum vulgarare* - wheat germ) in lung tissues and in Clara cell culture.

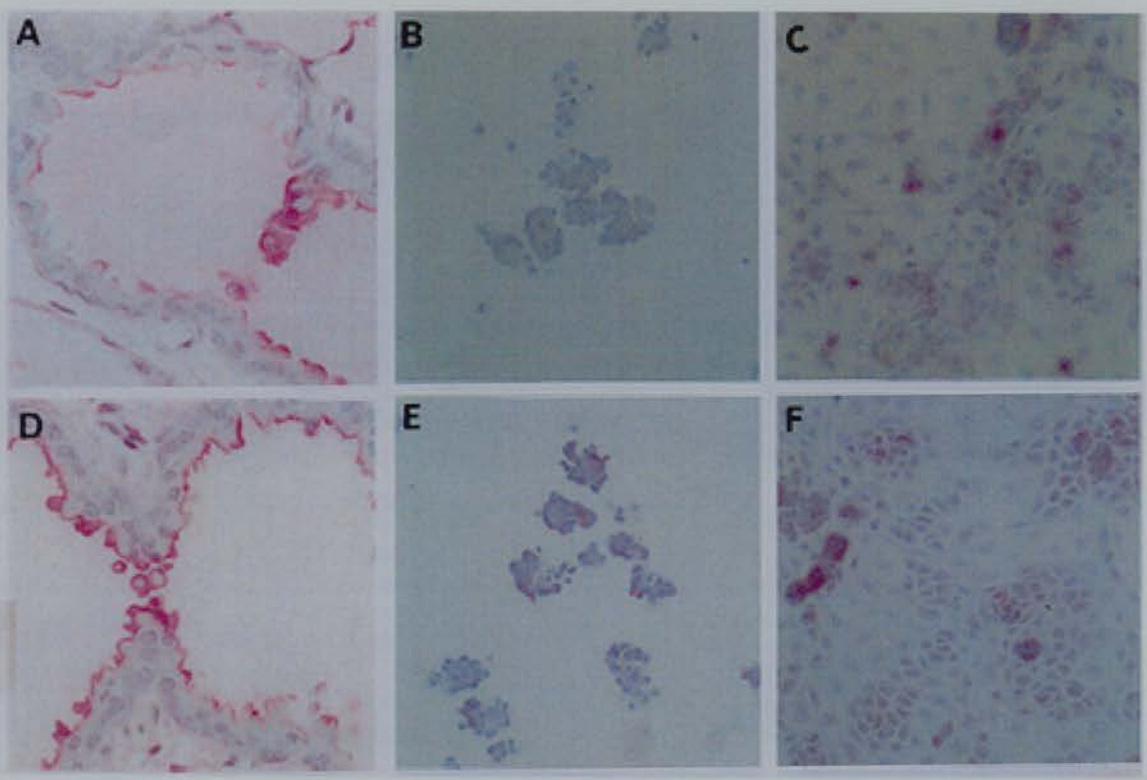

Figure 4.4 - Lectin binding studies using ABC Vector Red detection solution.

A Bauhinia purpurea (BPA) staining, Lung bronchiolar mouse tissue section, formalin fixed, magnification x400.

B BPA, freshly isolated Clara cells, formalin fixed, magnification x200.

C BPA, cultured Clara cells at day 5, formalin fixed, magnification x200.

D Maclura pomifera MPA staining, Lung bronchiolar mouse tissue section, formalin fixed, magnification x400.

E MPA, freshly isolated Clara cells, formalin fixed, magnification x200.

F MPA, cultured Clara cells at day 5, formalin fixed, magnification x200.

4.6 Cytokeratin expression in Clara cell cultures.

Keratin intermediate filaments are among the most differentiation-specific proteins synthesised in epithelial cells. More than 20 different cellular keratins (known as cytokeratins to distinguish from the keratins present in nails and hair) have been identified each of which appears to have a distinctive pattern of synthesis in normal epithelia (McBride et al., 1999; Mukhopadhyay and Roth, 1996; Gunning et al., 1992).

Approximately 90% freshly isolated Clara cells contained keratin 8 and 18, as detected immunocytochemically indicating their epithelial origin (Figure 4.5). At 24 hours and 120 hours in culture, all cells were keratin-positive suggesting that contaminating non-epithelial cells such as macrophages or fibroblasts present in initial isolated had either failed to attach or had failed to survive in culture.

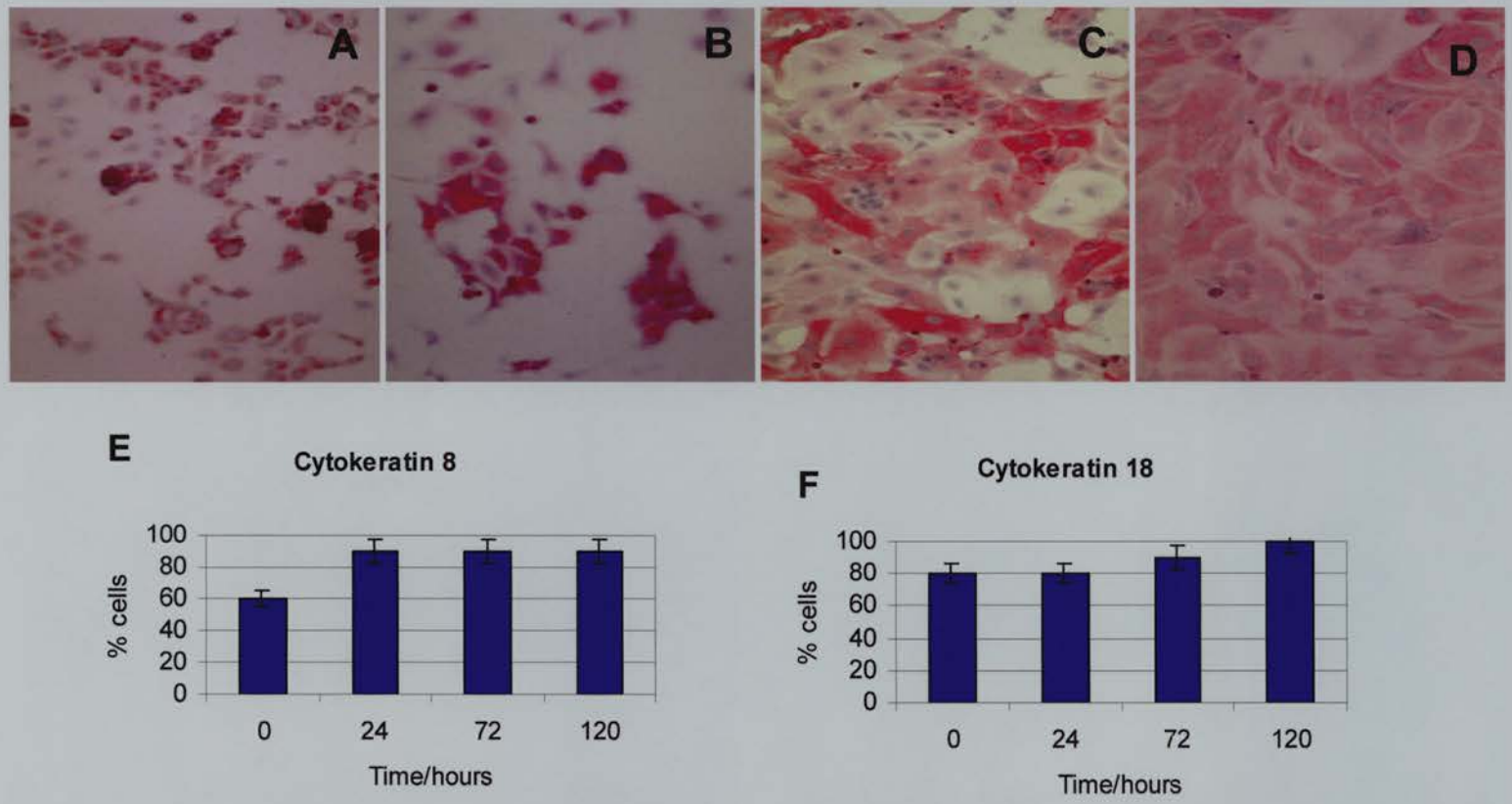

Figure 4.5 - Cytokeratin expression in primary Clara cell cultures. Red staining is the positive staining for cytokeratin using Vector Red™. Cells were counterstained with haematoxylin seen as light blue nuclei. From time 24 hours to 120 hours in culture, most cells were cytokeratin-positive suggesting few contaminating non-epithelial cells such as macrophages or fibroblasts (E & F). [(A) cytokeratin 8 expression at time 24 hours; (B, C & D) cytokeratin 18 expression at times 24, 72 and 120 hours respectively]. Magnification x200.

4.7 Changes in Clara cell ultrastructure upon culturing.

Electron microscopy (EM) probably remains the definitive method presently available to characterise a cell type. The organelles contained by a cell can be determined and localised and stained procedure can be carried out to identify material contained within a cell. The organelle complement of a cell is specific to the function of that cell and therefore, EM allows accurate diagnosis of cell type (Peão et al., 1993; Ten Have-Opbroek et al., 1991).

At time 0 hours in culture, 78% of the observed cells were Clara cells. The other 22% were almost all ciliated cells (n = 107). Clara cells were in good condition, although there were less granules than would perhaps be expected, and the smooth endoplasmic reticulum was not as distinct as might be anticipated. The prevalent feature of these cells was the abundance of mitochondria. A striking abnormal feature in many of both the Clara and ciliated cells is the presence of large vacuoles within the cytoplasm (Figure 4.6 A).

At time 24 hours in culture, 80% of the observed cells were Clara cells (n = 104). Morphologically these Clara cells were indistinguishable from the time 0 hours samples, the viable cells possessing an abundance of mitochondria but very few granules (Figure 4.6 B). As at time 0 hours, many of the cells contained a single, often large vacuole in the cytoplasm. At time 120 hours in culture, 85% of the observed cells were Clara cells (n = 100). There was little change in the cell purity and viability from 24 hours in culture perhaps indicating that once the initial increase in necrosis has occurred, the remaining cells stay viable. The vacuolation was again evident, but more obviously, both the nucleus and cytoplasm appeared more diffuse in these cells compared to 24 hours. Furthermore, there also appeared to be a drop in mitochondria size and number per cell compared to cells at 24 hours in culture (Figure 4.6 C).

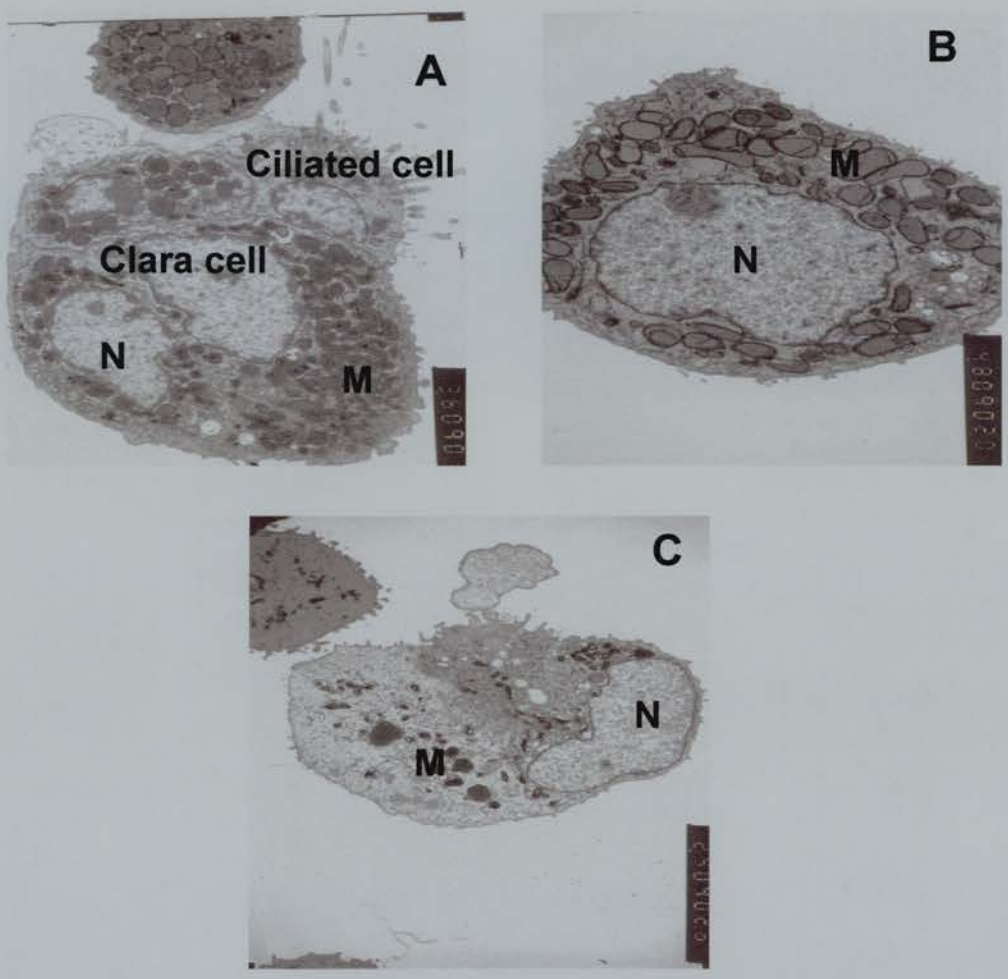

Figure 4.6 - Ultrastructure of Clara cells
(A) Freshly isolated Clara cells with a ciliated cell, magnification x 10,400. There was a decrease in the number of mitochondria (M) in Clara cells at 120 hours in culture (C) compared to Clara cells at 24 hours in culture (B). (N) is the cell nucleus. Magnifications: (B) x 13,900; (C) x 8,400.

4.8 Proliferation in Clara cell cultures.

Two populations of nuclei have been identified in Clara cell cultures: normal regular sized nuclei and abnormal larger nuclei (Figure 4.7B). From 24 hours to 120 hours in culture there seems to be an increase in the total of larger nuclei. From 24 hours to 72 hours in culture there was a decrease in the positive normal nuclei, while an increase in both the positive and negative larger nuclei. From 72 hours to 120 hours in culture the number of cells with positive normal nuclei remained the same, the percentage of cells with positive larger nuclei decreased while the number of cells with negative larger nuclei decreased (Figure 4.7 A). The total proliferation rate was about 10 % throughout the culturing period.

The larger nuclei may be the consequence of incomplete or partial cytokinesis in mitosis, a feature noted in liver cells by time lapse videomicroscopy (S. Prost, personal communication). This phenomenon is known as endoreduplication and is defined as an unscheduled round of DNA replication (Bates et al., 1998; Chang et al., 2000; Jiang et al., 2000). $p21^{Waf1/Cip1}$ can be an important factor leading to endoreduplication. Bates et al., (1998) have shown that $p21^{waf1/Cip1}$ can induce both a G1 arrest and G2 delay, but fails to significantly delay progress through S-phase in the p53/pRB null osteosarcoma cells line, Saos-2. $p21^{waf1/Cip1}$ expression significantly inhibited all cyclin dependent kinase activity and several of the $p21^{waf1/Cip1}$ expression resulted in endoreduplication in the G2 blocked cells.

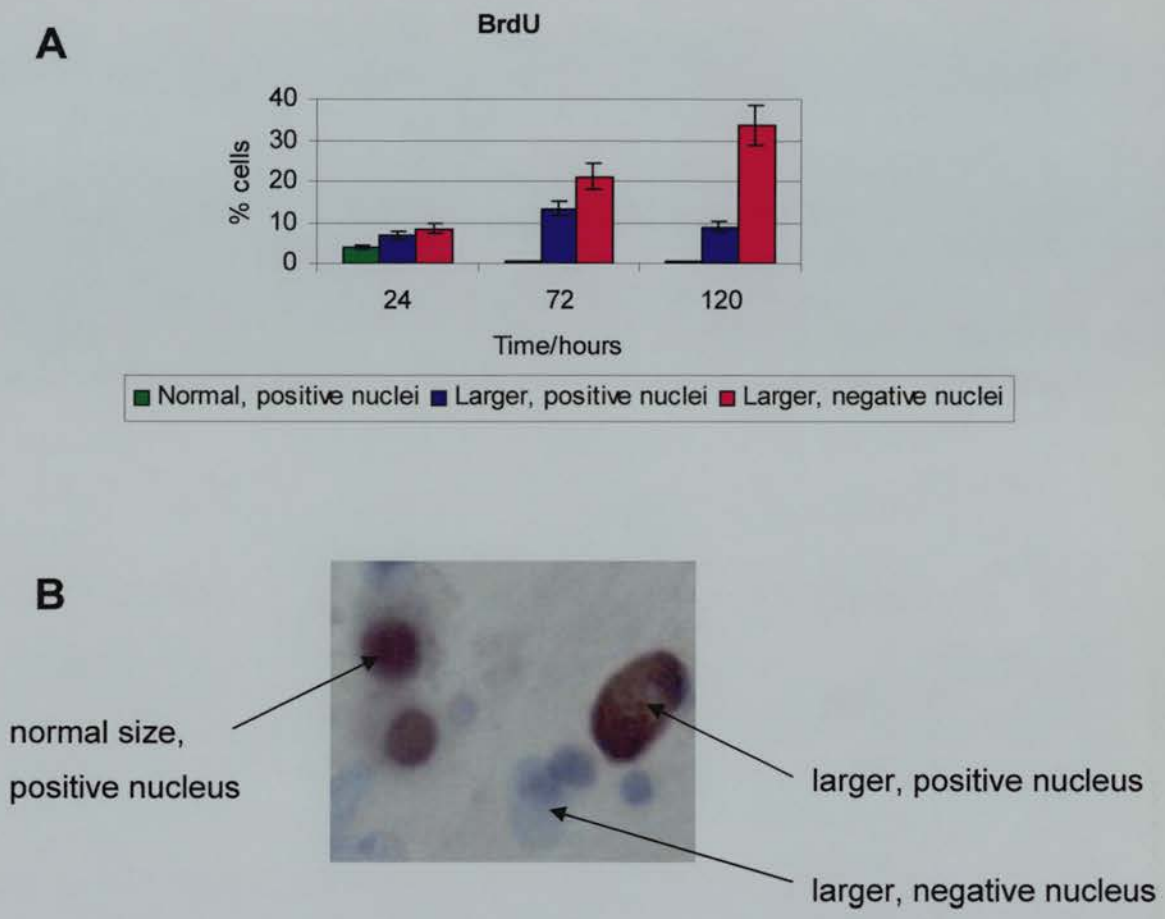

Figure 4.7 - BrdU incorporation in primary Clara cell culture. BrdU incorporated nuclei are stained brown, cells were counterstained with haematoxylin (blue nuclei). Over the culture period, the BrdU incorporation counts were relatively constant throughout the culture period (A). (B) represents a typical normal and larger positive nuclei. Magnification x400.

4.9 Feulgen staining

Feulgen staining is a method of quantifying DNA content. From the morphology and size of the nucleus, it is possible to determine whether a cell is in mitosis, apoptotic or necrotic stage.

Mitosis is an essential phase of the cell cycle, which leads to cell division. In mammalian cells, mitosis usually takes about an hour. During that period the cell first builds up and then breaks down a specialised microtubular structure, the mitotic apparatus, and structure larger than the nucleus that is designated to capture the chromosomes (prophase), align them (metaphase) and finally separate them and daughter chromosomes (anaphase) (Elledge, 1996; Grana and Reddy, 1995; Ji et al., 1997; Murakami and Nurse, 2000; Nurse et al., 1998; Sherr, 1996). Typical mitotic Clara cells are shown in figures 4.8 C & D.

The morphological changes in apoptosis have been extensively reviewed. In apoptotic cell, cells shrink in volume, lose contact with their neighbours and lose specialised surface elements such as microvilli and cell-cell junctions (Bellamy et al., 1995; Dowen, 1993; Guo and Hay, 1999; Haake and Polakowska, 1993; Kerr et al., 1972; Vaux et al., 1994; Vitale et al., 1999; Wyllie, 1987; Wyllie et al., 1998; Wyllie et al., 1994; Yamasaki, 1999). Apoptosis of Clara cells in primary culture is shown in figure 4.8 B.

Necrotic cells characteristically swell rather than shrink. Plasma membrane damage leads to a loss of calcium and water balance, which is followed by drop in pH (acidosis) and osmotic shock. The acidosis, leads to the general condensation of chromatin, leading to a darkened or 'pyknotic' nucleus. Both the inner and outer compartments of the mitochondria become distended and dense deposits of matrix lipoprotein appear. Finally the endoplasmic reticulum and lysosomes swell and burst (Born et al., 1999; Dwyer-Nield et al., 1998; Kanekal et al., 1990; Kerr, 1971; Sauer

et al., 1997; Shirakawa et al., 2001). A typical necrotic Clara cell is shown in figure 4.8A.

The mitotic rate of Clara cells throughout culturing from time 24 hours to 120 hours in culture was about 1%. This means that at any time point, 1% of the cells in culture undergo mitosis. No significant changes in necrosis or apoptosis rate were observed during Clara cell culture (Figure 4.8 E).

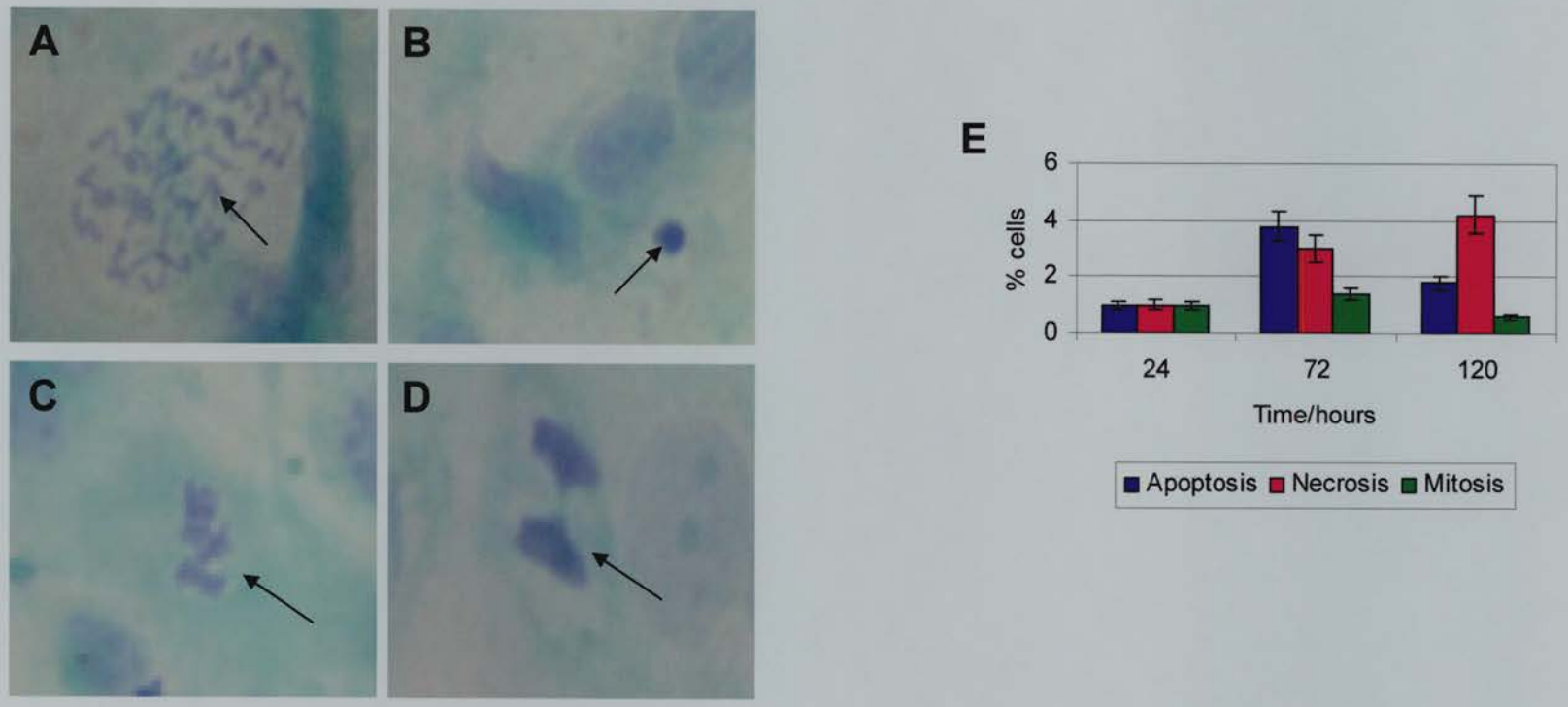

Figure 4.8 - Feulgen stain of primary Clara cell cultures. (A) Typical necrotic cell showing breakdown of entire nucleus and cytoplasmic content (arrow); (B) Typical apoptotic cells indicating an apoptotic body (arrow); (C &D) mitotic Clara cells at different stages in cell division (arrows). Magnification x 400. No significant changes in apoptosis, necrosis and mitosis were observed in Clara cells upon culture time (E).

4.10 Integrin characterisation.

Integrins are a family of cell-surface glycoproteins that act as receptors for ECM proteins, or for membrane-bound counter-receptors on other cells. Integrin-mediated cell-ECM adhesion sites are complex specialised structures termed focal contact or focal adhesions (Aplin et al., 1998). Each integrin is a heterodimer that contains an α and a β subunit with each subunit having a large extracellular domain, a single membrane-spanning region, and in most cases (other than β_4), a short cytoplasmic domain (Hynes, 1992; Hynes, 2000; Frisch and Ruoslahti, 1997). The integrin receptor family of vertebrates includes at least 16 distinct α subunits and at least 8 β subunits which can associate to form more than 20 distinct integrins (Hynes, 1992; Liapis and Hutton, 1997; Coraux et al., 1998; Frisch and Ruoslahti, 1997; Ingber, 1991; Mizejewski, 1999; Schwartz, 1997).

The integrins expressed in diverse cell types display different ligand specificities. In addition, during dynamic and complex processes, such as organ development and tumour progression and metastasis, the cellular distribution and/or intensity of expression may change (Giancotti, 1997; Arroyo et al., 2000; Boudreau and Jones, 1999; Brown, 2000; Coraux et al., 1998; Dedhar and Hannigan, 1996; Felding-Habermann et al., 2001; Frisch and Ruoslahti, 1997; Giancotti, 1997; Hemler, 1998; Howe et al., 1998; Hynes, 1992; Ingber, 1991; Legier et al., 2001; Schwartz, 1997; Shyy and Chien, 1997).

There are no published reports describing integrin characterisation in mouse lung tissue and mouse Clara cells. Six integrins were investigated mainly α_5, α_v, α_6, β_1, β_3 and β_4. Most of the antibodies, mainly α_5, α_v, α_6, β_1, and β_3 were obtained as a gift from Prof. Hideo Yagita, Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan while β_4 antibody was commercially available from Chemicon. The β_1 antibody obtained from Prof. Yagita, is now commercially

available from PharMingen (Catalogue No. 553837) and this is a functional β_1 blocking antibody (Burns et al., 2001; Noto et al., 1995).

Various conditions were evaluated in order for the detection to be successful. The best results were obtained when the microwave antigen retrieval method (section 2.2.1.2.2) was used on methocarn (Appendix 1) fixed tissue sections. The use of microwave heating on formalin-fixed tissue for integrin detection have not been successful (Cattoretti et al., 1993), with the exception of a relatively recent report (Liapis and Hutton, 1997; Gladson et al., 1996). Liapis and Hutton, (1997) were still unable to detect specific immunoreactivity with several antibodies they used. A number of alternative buffers (Alsbeh and Battifora, 1995), varying the buffer pH (Grossfeld et al., 1996), or use of newly developed tissue fixatives (Muller et al., 1996)have been described. Even though the exact mechanism through which microwave heating works is still unclear (Shi et al., 1995; Shi et al., 1991), signal detection for many commonly used antibodies is superior compared to other methods such as autoclave or water bath (Tani and Phillips, 1995).

α_v, α_6, β_1, and β_4 were all present in both mouse lung tissue sections and in primary Clara cell culture. The integrin β_3 was absent in both mouse lung tissue (figure 4.9) and primary Clara cell culture (figure 4.10; table 4.2). Although various modifications were carried out (as explained above), the integrin β_3 was still absent. The absence β_3 integrin in mouse lung and Clara cells could be either due to the non-reactive nature of the antibody or because the integrin β_3 was not present. The latter hypothesis is more plausible since β_3 integrin is absent in the human bronchiolar region (Pilewski et al., 1997).

	alpha 6	alpha 5	alpha v	beta 1	beta 3	beta 4
tissue sections	++	++++	+++	+++	0	++++
24 hours	++	++++	+++	+++	0	++++
72 hours	+	+++	+++	+++	0	++++
120 hours	+	++++	+++	+++	0	++++

Key:
- ++++ very strong positive
- +++ strong positive
- ++ moderately positive
- + weak positive
- ± very weak positive
- 0 no stain

Table 4.2 - Integrin characterisation

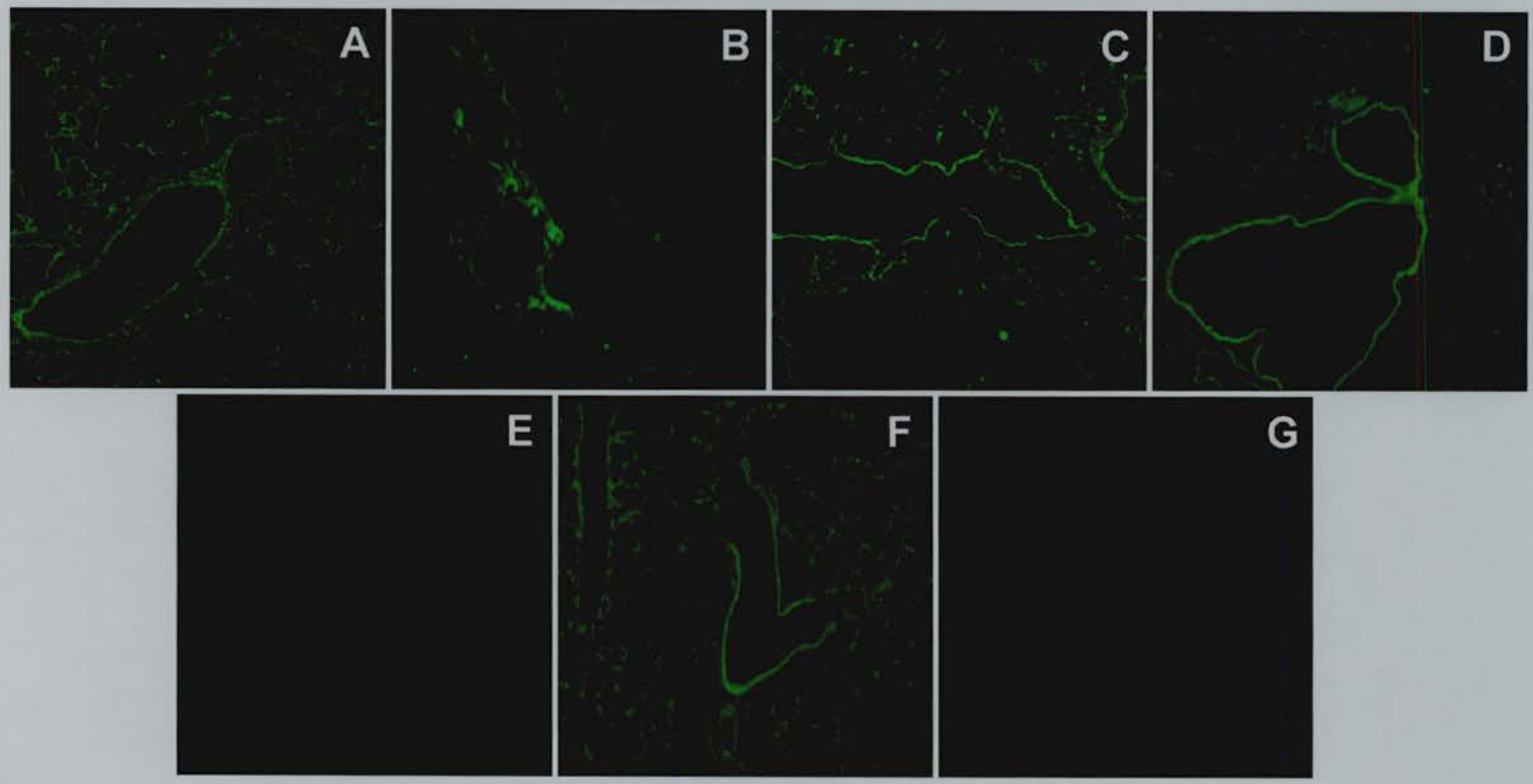

Figure 4.9 – Integrin characterisation in mouse bronchiolar region of lung tissue sections using Alexa™ 488 conjugated secondary antibody. Integrins alpha 5, alpha v, alpha 6, beta 1 and beta 4 were present in mouse small airways (A, B, C, D & F respectively, while integrin beta 3 was absent (E). G shows a typical negative control whereby the primary antibody was omitted. Magnification x 200.

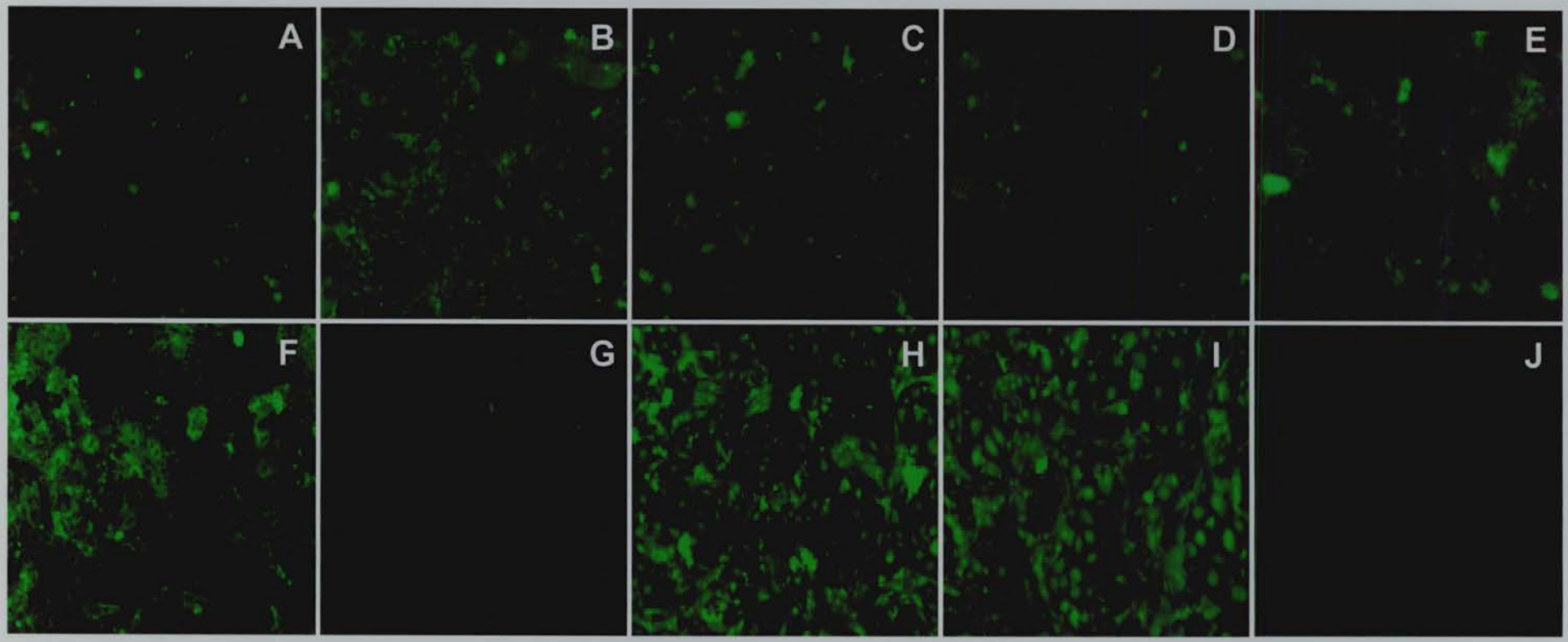

Figure 4.10 - Integrin characterisation in Clara cell cultures using Alexa^{TM} 488 conjugated secondary antibody. An increase in the expression of alpha v integrin was observed in Clara cells from 48 hours (A) to 72 hours (B) in culture. A decrease in the expression of alpha 6 was observed from 24 hours (C) to 120 hours (D). No changes in the expression of integrins alpha v (E), beta 1 (F) and beta 4 (H & I). Beta-3 integrin was not expressed in primary Clara cells at all time points (G). [(E, F, I & G) are Clara cells at 120 hours in culture; (H) Clara cells at 72 hours]. J is the negative control whereby the primary antibody was omitted. Magnification x 200.

4.11 Proliferating Cell Nuclear Antigen (PCNA) expression.

Proliferating cell nuclear antigen (PCNA) is an acidic nuclear non-histone protein whose rate of synthesis is modulated during the cell cycle. It increases during S-phase and correlates directly with the proliferative state of normal cells (Bravo and Celis, 1985; Bravo et al., 1981; Bravo et al., 1987; Bravo and Macdonald-Bravo, 1987; Bravo and Macdonald-Bravo, 1985; Tanno et al., 1996; Tanno and Taguchi, 1999). PCNA is required for both DNA replication and DNA repair (Li et al., 1995a; Moriuchi, 1990; Oku et al., 1998; Rossi et al., 1999; Rousseau et al., 1999; Yu et al., 2001). Although the total cellular level of PCNA is relatively constant through the cell cycle, it is tightly associated with the nucleus only in S-phase cells and in UV-irradiated non-S-Phase cells (Bravo and Macdonald-Bravo, 1987; Bravo et al., 1981). There are three forms of PCNA, the L and H types are found in the nucleus, while the P and L types are found in the cytoplasm. The L-type (eluted at low concentrations of KCl from a phosphocellulose column) and H type (eluted at high KCl concentrations) were observed in the nucleoplasm of regenerating livers 24 and 27 hours after surgery. PCNA in the cytoplasm are P type (eluted in the pass through fraction) and L type. Surprisingly, the total amounts of P type and L type in the cytoplasm extracts are comparable to those of L type and H type in nucleoplasm. This data suggests that newly synthesised PCNA is immediately converted into the P and L complex forms. The P type and some of the L type that lacks a nuclear localisation signal remain in the cytoplasm, the rest of the L type with nuclear localisation signal is transferred to into the nucleus (Tanno and Taguchi, 1999).

In the mouse lung bronchiolar region about 10% of the cells were nuclear PCNA positive while about 40% cytoplasmic PCNA positive (Figure 4.11A). From 24 hours to 120 hours in culture, a decrease ($p < 0.05$) in cytoplasmic PCNA while an increase ($p < 0.05$) in nuclear PCNA expressions were observed (Figures 4.11 B, C, D & E).

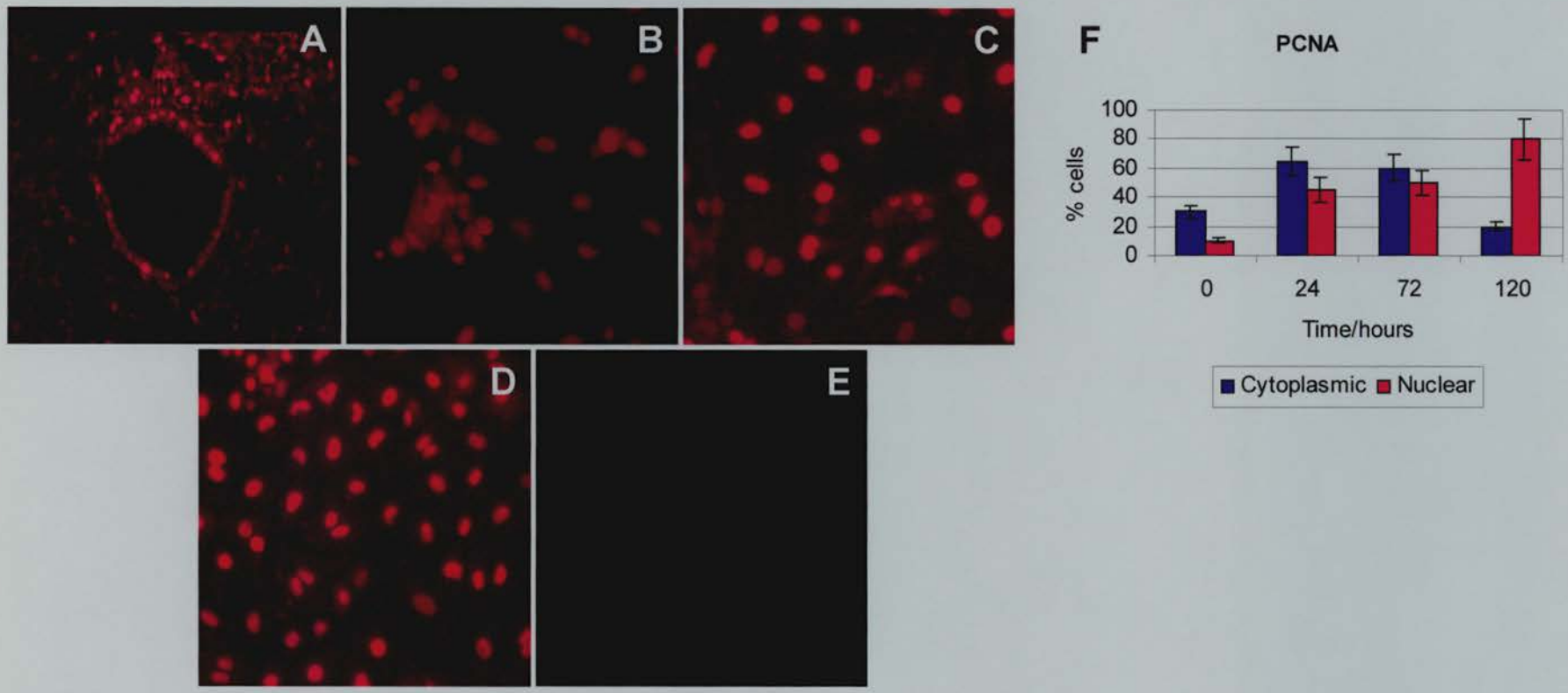

Figure 4.11 - PCNA expression in the lung bronchiolar region and in primary Clara cell cultures using Alexa™ 594 (red) conjugated secondary antibody. In the mouse bronchiolar region, about 10% of the cells express nuclear PCNA while about 40% of the cells express cytoplasmic PCNA (A). From time 24 hours (B) to 72 hours (C) to 120 hours (D) in Clara cells culture, there is an increase in nuclear PCNA expression while a decrease in cytoplasmic PCNA expression (F). E is the negative control, whereby primary antibody was omitted. Magnification x200.

4.12 p21 expression.

$p21^{WAF1/Cip1}$ belongs to the Cip/Kip family of CKIs ($p21^{Cip1/Waf1}$, $p27^{Kip1}$, $p57^{KIP2}$) (Elledge, 1996; Murakami and Nurse, 2000; Nigg, 1995; Nurse et al., 1998). The functions of p21 are not yet fully understood but it is known that p21 is involved in cell proliferation, differentiation and death (Sheikh et al., 1997; Cayrol et al., 1998; Li et al., 1995a; Asada et al., 1999; Bates et al., 1998; Bulavin et al., 1999; Butz et al., 1998; Cai and Dynlacht, 1998; Clarke et al., 1995; Costanzi-Strauss et al., 1998; Donato and Perez, 1998; Dotto, 2000; Howe, 2001; Jabbur et al., 2000; Lu et al., 1998; McKay et al., 1998; Rancourt et al., 2001; Sheikh et al., 1997; Tchou et al., 1996; Wang et al., 2000; Wang et al., 1999; Wu et al., 1998a; Yamamoto et al., 1998; Zhang et al., 1999). p21 was shown to promote apoptosis, protect cells from undergoing apoptosis and inhibits differentiation (Oku et al., 1998; Rousseau et al., 1999; Asada et al., 1999; Bulavin et al., 1999; Clarke et al., 1995; Donato and Perez, 1998; Gervais et al., 2000; Jiang et al., 2000; Lu et al., 1998; McKay et al., 1998; Wu et al., 1998a;Zhang et al. 1999).

p21 was also found to be in the cytoplasm and various different form/sizes were described including 21kDa, 20kDa, 19kDa, 16kDa, 15kDa and 14kDa (Tchou et al., 1996; Asada et al., 1999; Donato and Perez, 1998; Poon and Hunter, 1998; Orend et al., 1998; Gervais et al., 2000; Zhang et al., 1999). The functional role of cytoplasmic p21 is not clear. Asada et al., (1999) showed that cytoplasmic p21 complexes with ASK1 and inhibits apoptosis whereas Zhang et al., (1999) showed that cytoplasmic $p21^{Waf1/Cip1}$ converts cancer cells from growth arrest to undergo apoptosis.

In a normal mouse bronchiole, 1-2% of the cells were positive for nuclear p21, but most of the cells had cytoplasmic staining (Figure 4.12 A & B). No significant increase in the expression of cytoplasmic and nuclear p21 in Clara cells cultures from 24 hours to 120 hours in culture.

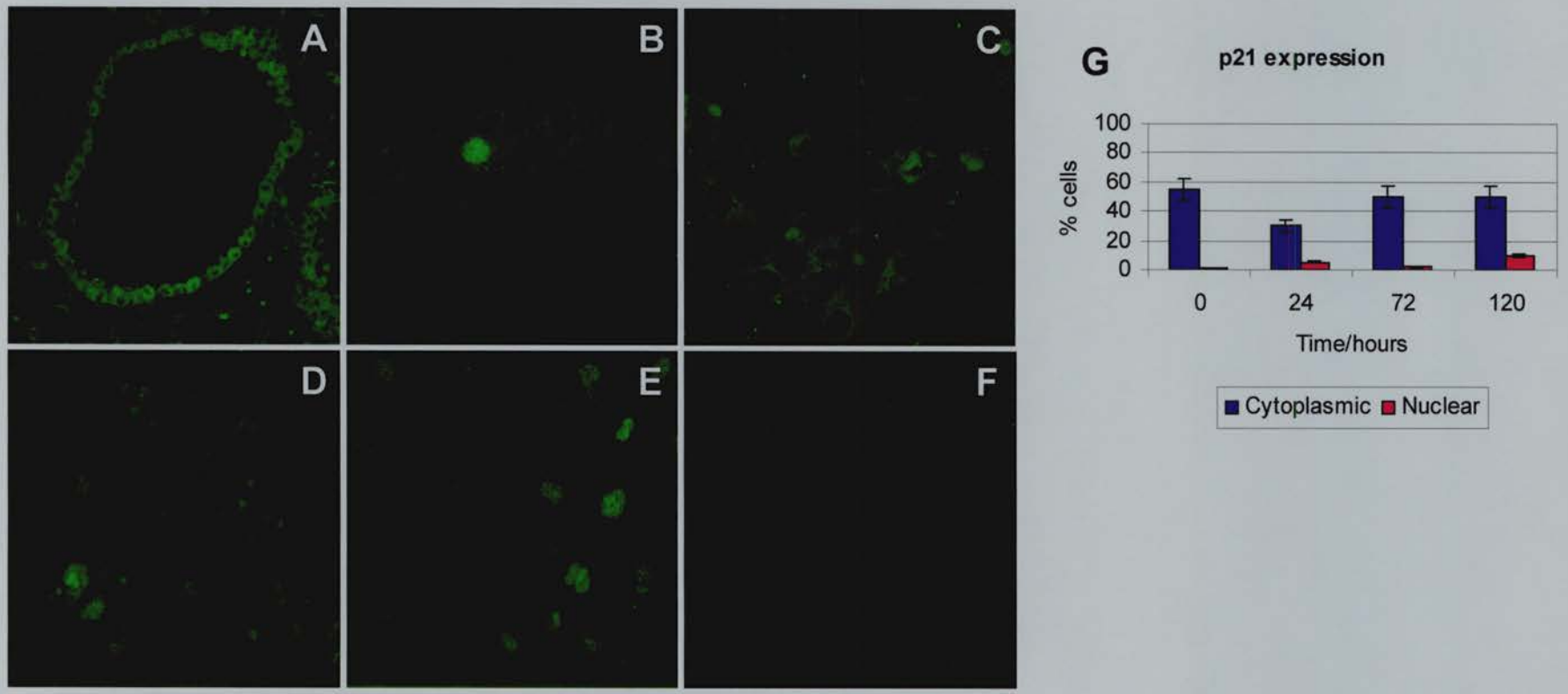

Figure 4.12 - Expression of p21 in lung bronchiolar region and in primary Clara cell cultures. In the mouse bronchiolar region only about 1-2% of the cells have nuclear p21 expression, but most of the cells have cytoplasmic p21 (A & B). No significant changes in the expression of cytoplasmic and nuclear p21 was observed an Clara cell cultures at 24 hours (C), at 72 hours and at 120 hours (E). F represents a typical negative control, whereby the primary antibody was omitted. Magnifications of (A, C, D, E & F) x 100, Magnification of B x 400.

4.13 p53 expression.

The p53 proteins are potent inhibitor of cell growth, able to arrest the cell cycle at several points and under some circumstances activate the apoptotic machinery leading to cell death (Brambilla and Brambilla, 1997; Hupp et al., 2000; Ji et al., 1997; Jimenez et al., 1999; Kaelin, 1999b; Kamijo et al., 1998; Lakin and Jackson, 1999; Lane, 1992; May and May, 1999; Morgenbesser et al., 1994; Sigal and Rotter, 2000; Stewart et al., 2001; Vaziri and Benchimol, 1999; Vitale et al., 1999; Wyllie et al., 1994; Yap et al., 1999). Although p53 gene mutation or deletions occur in a substantial proportion of human cancers, many tumours contain p53 that is functionally inactivated through other mechanisms. One such mechanism involved aberrant sub-cellular localisation. Cytoplasmic localisation of wildtype p53 has been reported in inflammatory breast carcinoma, colorectal adenocarcinoma, undifferentiated neuroblastoma (Ostermeyer et al., 1996; Moll et al., 1995), hepatocellular carcinoma and retinoblastoma (Morgenbesser et al., 1994). p53 cytoplasmic sequestration could result from its anchorage to a cytoplasmic tether or by an imbalance in nuclear-cytoplasmic shuttling (Jimenez et al., 1999). The functional significance of cytoplasmic p53 is still unclear.

In the mouse bronchiolar region no cells were nuclear positive for p53 immunohistochemistry, but most of the cells had cytoplasmic p53 as determined by confocal microscopy. No significant changes in the expression of both cytoplasmic and nuclear p53 were observed in cultured Clara cells (figure 4.13).

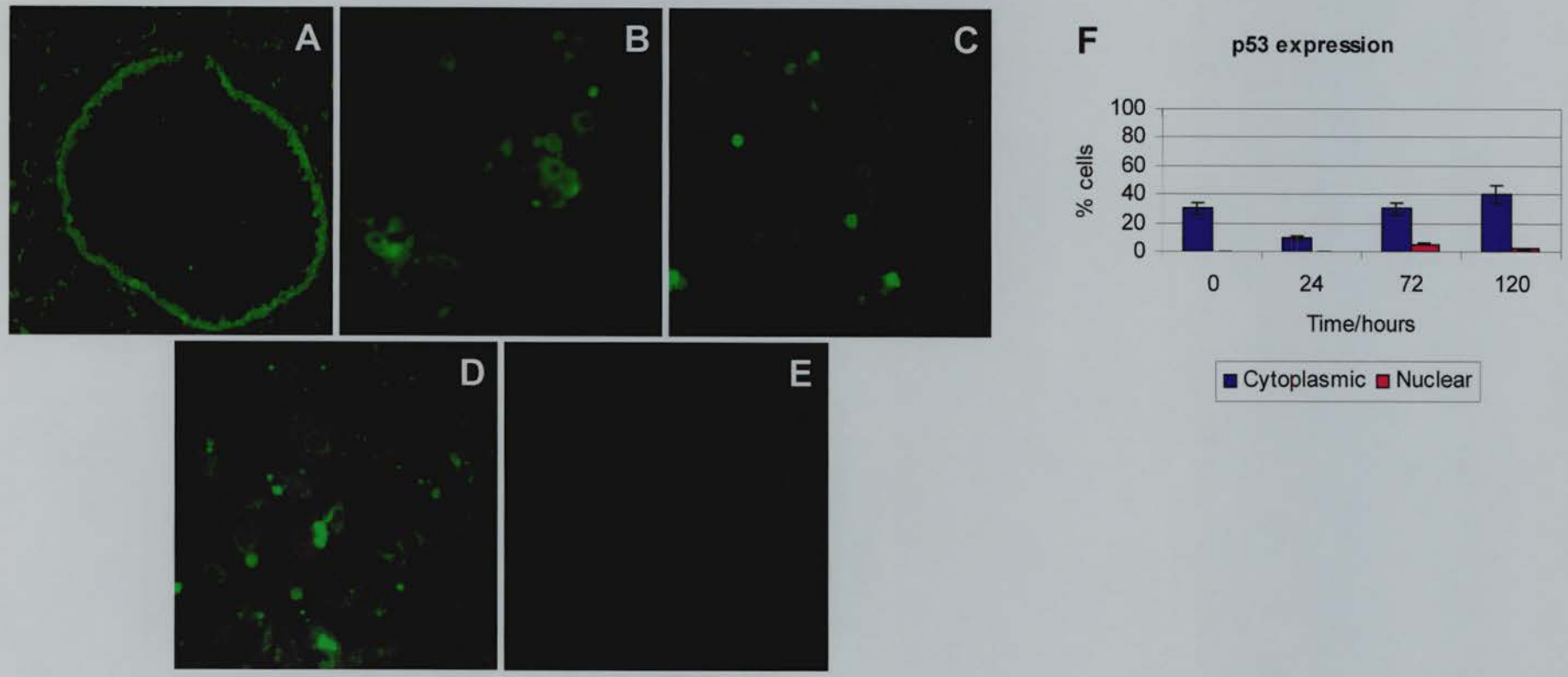

Figure 4.13 - p53 expression in mouse lung bronchiolar region and in cultured primary Clara cells. Nuclear p53 expression was absent in lung bronchiolar region (A). No significant changes in the expression of nuclear and cytoplasmic p53 were observed over culture period (F) at 24 hours (B), at 72 hours (C) and at 120 hours (D). E is a typical negative control, whereby the primary antibody was omitted. Magnification x 200.

4.14 Epithelial growth factor receptor (EGF-R) expression.

Growth factors regulate the growth and development of many organs, including lungs. Most growth factors signal their mitogenic activities through tyrosine kinase receptors. Epithelial growth factor receptor (EGF-R) is perhaps the best studied member of this larger receptor family (Miettinen et al., 1997). EGFR consists of a large cysteine-rich extracellular domain, a single transmembrane segment, and a long cytoplasmic domain, which contains the 11 subdomains characteristic of kinases (Schlessinger and Ullrich, 1992; Ullrich et al., 1984). A number of structurally related ligands including EGF, TGF-α, HB-EGF, amphiregulin, and beta cellulin, bind to the EGF-R (Miettinen et al., 1997; Ohtsubo et al., 1998; Oksvold et al., 2000; Sibilia and Wagner, 1995; Skarpen et al., 1998; Taupin et al., 1999; Threadgill et al., 1995; Wu et al., 1999). Ligand binding leads to receptor dimerisation and autophosphorylation of the kinase domain, followed by recruitment of cytoplasmic effector proteins through their SH2 domains to the tyrosine-phosphorylated sites, initiates signalling cascades which lead to cell proliferation and differentiation (Schlessinger and Ullrich, 1992).

At high concentrations EGF inhibits growth in A431 cells. Growth suppression at high concentrations of EGF was demonstrated to correlate with increased expression of the cyclin-dependent kinase (CDK) inhibitor $p21^{Kip1/Cip1}$. At growth stimulatory concentrations of EGF increased levels of $p21^{Kip1/Cip1}$ were not observed (Skarpen et al., 1998; Haugh et al., 1999). Stimulation of EGF-R was also observed to cause activation of STAT 1 and STAT 3 and activated STAT 1 has been shown to induce $p21^{Kip1/Cip1}$ and thereby inhibit proliferation of A431 cells (Grandis et al., 1998; Coqueret and Gascan, 2000).

Ligand-bound EGF-R is rapidly internalised through receptor-mediated endocytosis and sorted to multivesicular bodies. The receptor either recirculates to the plasma

membrane after ligand dissociation or is degraded through the lysosomal pathways (Oksvold et al., 2000). Ligand-activated and internalised EGF-R appears to be phosphorylated and adaptor protein-associated, but the importance and function of endosomal signal transduction is controversial (Oksvold et al., 2000; Skarpen et al., 1998; Di Guglielmo et al., 1994; Emlet et al., 1997; Futter et al., 1993; Lai et al., 1989; Nesterov et al., 1994).

In mouse bronchiolar region most of the cells expressed EGF-R on the plasma membrane but no endocytosed EGF-R staining was visible. EGF-R expression was higher in unattached cells (freshly isolated Clara cells at time 0 hours) compared to cultured cells at time 24, 72 and 120 hours. No significant changes in the expression of EGF-R were observed though the culture period (Figure 4.14).

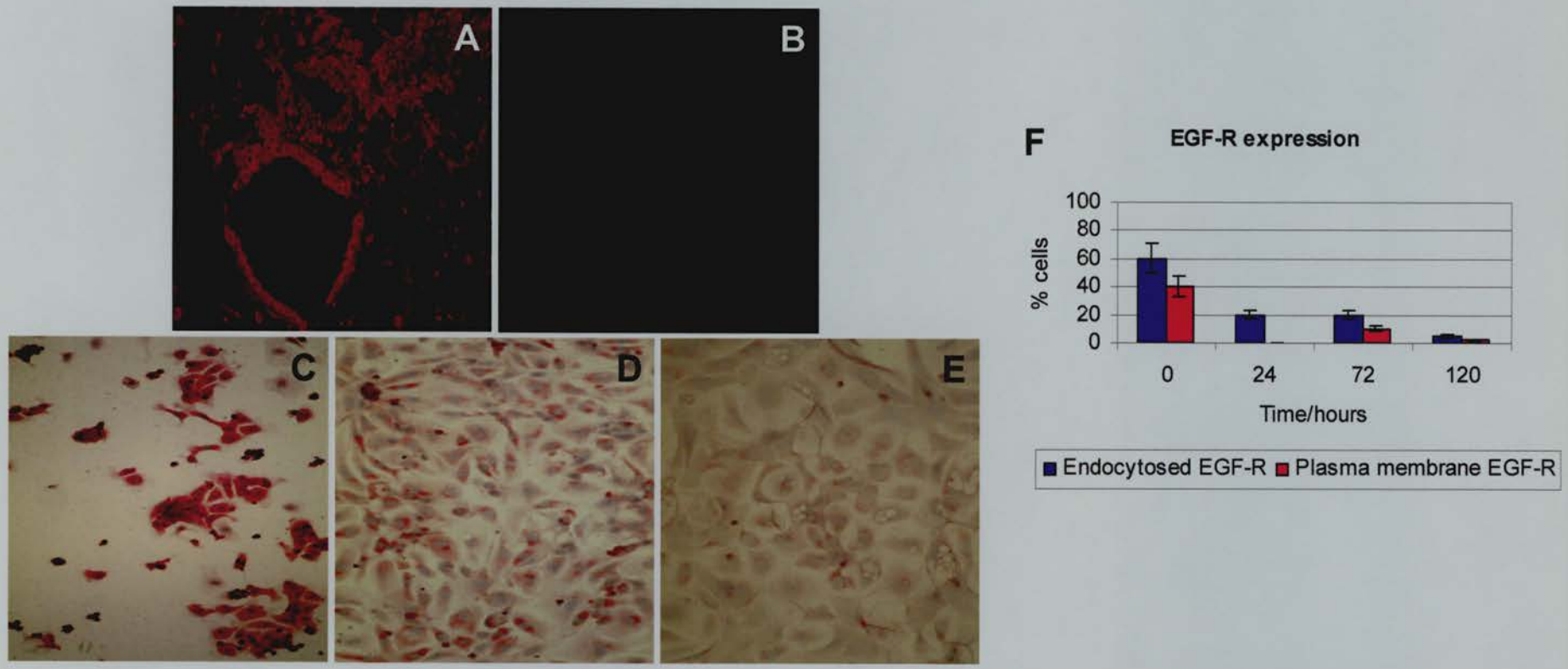

Figure 4.14 - EGF-R expression in mouse lung bronchiolar region and in primary Clara cell cultures. In the mouse lung bronchiolar region, EGF-R was expressed in the plasma membrane while no endocytosed EGF-R was expressed (A). EGF-R expression both endocytosed and plasma membrane was higher in unattached freshly isolated Clara cells at time 0 hours (C) compared to cultured Clara cells (D, E & F). No significant changes in the expression of EGF-R was observed through culturing (D & E). [D & E are Clara cells at 72 and 120 hours in culture respectively]. B represents a typical negative control for immunofluorescence whereby the primary antibody was omitted. Magnification x 200.

4.15 $p27^{Kip1}$ expression.

The cyclin-dependent kinase (Cdk) inhibitor $p27^{Kip1}$ is an important regulator of the mammalian cell cycle ((Resnitzky et al., 1995; Reed et al., 1994; Sherr, 1996; Yamamoto et al., 1998; Lloyd et al., 1999). p27 negatively regulates G1 progression by binding to cyclin-Cdk2 complexes and preventing their activity. Both p27 and p21 form complexes with cyclin-Cdk4/6 and positively regulate the cell cycle (Yamamoto et al., 1998; Orend et al., 1998; Lloyd et al., 1999; Harvat et al., 1998; Cheng et al., 1999).

The activity of p27 depends on its concentration, its distribution among cellular complexes and its subcellular localisation (Ekholm and Reed, 2000; Slingerland and Pagano, 2000; Rodier et al., 2001; Ishida et al., 2000). p27 needs to be transported into the nucleus to have its inhibitory action (Slingerland and Pagano, 2000; Lloyd et al., 1999; Orend et al., 1998; Tomoda et al., 1999). The regulation of p27 cellular localisation is poorly understood and the function of cytoplasmic p27 still remains unknown.

In mouse lung bronchiolar region about 2% of the cells were nuclear p27 positive, but most of the cells were cytoplasmic p27 positive. The cytoplasmic p27 expression was lower ($p < 0.05$) in freshly isolated Clara cells at time 0 compared to cultured cells at time 24, 72 and 120 hours. No significant changes in the expression of p27 both cytoplasmic and nuclear were observed during the culture period (Figure 4.15).

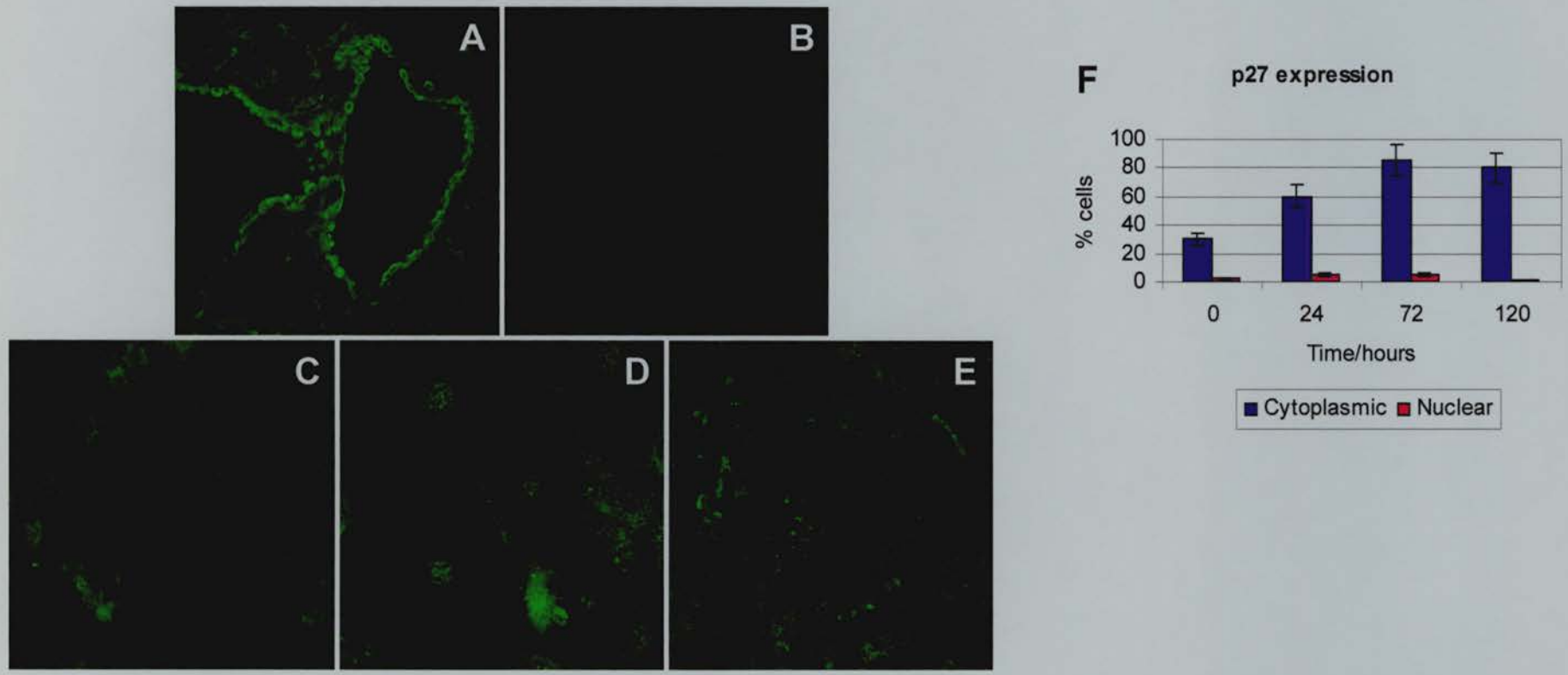

Figure 4.15 **p27 expression in mouse lung bronchiolar region and in primary Clara cell cultures. p27 was predominantly expressed in the cytoplasmic in the mouse lung bronchiolar region (A). No significant changes in the expression of nuclear p27 (F) was observed in cultured Clara cells at times 24 hours (C), 72 hours (D) and 120 hours (E). (B) is a typical negative control whereby the primary antibody was omitted. Magnifications: (A) x 200, (B to E) x 400.**

4.16 Discussion

4.16.1 Isolation and characterisation of Clara cells.

Clara cells have been successfully isolated and cultured in serum free medium for up to 120 hours. Most of the cells were healthy looking Clara cells, as determined by CC10 but some of the cells might have lost their functionality as seen by the decrease in the NBT assay.

From the lectin binding studies, it can be concluded that when Clara cells were grown on fibronectin (FN) and cultured in basal condition, cells dedifferentiate. The lectin binding in unattached freshly isolated cells at time 0 hours was different from cultured cells. This could be attributed to either cell injury during the digestion or due to cell-matrix disruption during the isolation. By time 24 hours in culture, cells seem to have recovered from the minor damage occurred during the isolation.

Most cells were cytokeratin positive which indicates that few non-epithelial cells were present. This observation was also confirmed by electron microscopy.. From 0 hours to 24 hours in culture there seem to be a slight increase in necrotic cells, this could be attributed due to cell attachment to the matrix. The number of mitochondria dropped from 24 hours to 120 hours in culture and there was also an increase in the number of vacuoles inside the cells. The actual significance of the decrease in the number of mitochondria is still unclear.

Six integrins were investigated alpha 5, alpha v, alpha 6, beta 1, beta 3 and beta 4. Alpha 5, alpha v, alpha 6, beta 1 and beta 4 integrins were present in Clara cells both in tissue sections and cultures. Beta 3 integrin was found to be absent in mouse Clara cells. Beta 3 integrins have been previously described to be absent in human bronchiolar epithelium (Damjanovick et al., 1992; Pilewski et al., 1997). During the culture there seems to be a decrease in alpha 6 from 24 hours to 120 hours in culture.

From 24 hours to 72 hours in culture there was a decrease in alpha 5 integrin expression.

4.16.2 Cell cycle progression in Clara cells.

Two populations of nuclei were observed, normal regular sized and larger nuclei. Although the proliferation rate as assessed by BrdU incorporation was constant from 24 hours to 120 hours in culture, there seems to be more proliferating cells having the larger abnormal nuclei rather than normal nuclei. One of the suggestions for this could be that the cell cycle was stopped at the cytokinesis stage thus the two nuclei did not separate. One could also hypothesise that p21 is involved since it has been shown previously that p21 could lead to cell cycle arrest and DNA endoreduplication (Chang et al., 2000; Bates et al., 1998; Jiang et al., 2000), but further studies need to be carried out.

The mitotic rate was about 1% throughout the culture period, as determined by Feulgen staining. The average BrdU incorporation in the Clara cell culture was about 10% throughout culture period. The proliferation rate in the lung bronchiolar region of both mammals (Ayers and Jeffery, 1988; Ji et al., 1995) and humans (Boers et al., 1998; Boers et al, 1999) was estimated to be about 1%.

From 24 hours to 120 hours in culture, there a decrease ($p < 0.05$) in cytoplasmic PCNA expression, but an increase ($p < 0.05$) in nuclear PCNA expression was observed. PCNA is known to be involved both cell proliferation and repair (Bravo et al., 1985; Bravo and Celis, 1985; Moriuchi 1990; Hall et al, 1995 Kelman 1997). Although there was an increase in PCNA at 120 hours in culture, there was no increase in either BrdU positive nuclei or mitotic index. This finding could be attributed to a growth arrest at the G1 phase or DNA endoreduplication suggesting that p21 might be involved. p21 can associate with PCNA, a subunit of DNA polymerase δ and can inhibit DNA replication directly, without affecting DNA repair

(Li et al., 1995a; Cayrol et al., 1998; Oku et al., 1998; Rousseau et al., 1999; Stillman 1996; Funk et al., 1997).

In normal bronchiole only a small number of cells have a positive nuclear staining for p21 (about 1-2%), but most of the cells have cytoplasmic staining. Similarly, none of the cells expressed nuclear p53, but most of the cells expressed cytoplasmic p53. No significant changes in the expression p53 and p21 (both cytoplasmic and nuclear) were observed in cultured Clara cells.

No significant changes in the apoptotic and necrotic rates were observed during Clara cell cultures. This could be due the constant expression of both p21 and p53. The actual role of cytoplasmic p21 is still unclear, but it has been shown that cytoplasmic p21 is involved in apoptosis (Asada et al., 1999). Further studies have to be carried out to determine the functional role of cytoplasmic p21 in Clara cells.

EGFR is one of the important receptors by which p21 can be up regulated or stabilised (Miettinen et al., 1997; Oksvold et al., 2000; Sibilia and Wagner, 1995; Skarpen et al., 1998; Threadgill et al., 1995; Wu et al., 1999). No significant changes in the expression of EGF-R were observed in Clara cell cultures, and as expected the p21 expression was constant through the culture period.

p27 is a CKI which is in the same family as p21. (Harvat et al., 1998; Juan et al., 1998; Lloyd et al., 1999; Moro et al., 1998; Orend et al., 1998; Resnitzky et al., 1995; Yamamoto et al., 1998). In normal mouse bronchiolar region and in cultured Clara cells, p27 was predominantly expressed in the cytoplasm. No significant nuclear p27 expression was observed upon Clara cell culture. A lower expression of cytoplasmic p27 ($p < 0.05$), was observed in unattached freshly isolated cells at time 0, indicating that lack of cell attachment or the disruption of cell-matrix interactions could lead to changes in the cell cycle regulation. Thus, further studies have to be carried out to study this hypothesis.

4.16.3 Conclusion.

Functional Clara cells have been isolated and cultured in serum free medium for up to 120 hours. Clara cells express alpha 5, alpha v, alpha 6, beta1 and beta 4 integrins but beta 3 subunit was absent in both tissue sections and cultured cells.

The isolated Clara cells seem to be quite constant throughout the culture period from 24 hours to 120 hours in terms of differentiation, proliferation and death. The expression of p21, p27 and p53 was constant throughout the culture period. Disruption of the cell-matrix interaction seems to influence the cell cycle progression, thus the ECM combinations could influence the cell fate. Further studies need to be carried out to manipulate the expression of p21 and to understand why the p21 expression in some diseases was lower while in other disease was higher.

Chapter 5 Effect of ECM on Clara cell proliferation, differentiation and death through p21 regulation.

5.1 Introduction

Extracellular matrix (ECM) is a general term that encompasses components of the basement membrane and interstitial connective tissue. The extracellular matrix contains signals that control cell shape, migration, proliferation, differentiation, morphogenesis and survival (Lukashev and Werb, 1998; Boudreau and Jones, 1999; Streuli, 1999).

After an injury to the lung epithelial cells, changes in the ECM composition could be a key regulator in restoring the epithelial barrier otherwise the injury could progress into a disease (Roskelley et al., 1995; Lukashev and Werb, 1998; Dunsmore and Rannels, 1996; Chintala and Rao, 1996; Talpale and Keski-Oja, 1997; Boudreau and Jones, 1999; Streuli, 1999; Ebihara et al., 2000). Cell-matrix interactions or disruptions affect the cell cycle regulation in various ways. A number of studies have shown that by the disruption of cell-matrix interactions, regulated cell cycle progression and influences the expression of a number of cell cycle regulatory proteins such as p21, p27 and p53 (Guadagno et al., 1993; Assoian, 1997; Schwartz and Assoian, 2001; Bao et al., 2002; Ilic et al., 1998; Nagaki et al., 2000; Wu and Schönthal, 1997). Other studies have shown that cell-matrix disruption could lead to apoptosis (Ruoslahti and Reed, 1994; McGill et al., 1997; Frisch and Francis, 1994; Bourdoulous et al., 1998; Kettritz et al., 1999; Sethi et al., 1999; Day et al., 1997).

The main hypothesis of this chapter is that cell-matrix interaction or disruption regulates cell cycle progression through p21. To study this hypothesis Clara cells from both wt and p21 ko mice were isolated and cultured on seven different ECM compositions: Fibronectin/Collagen IV/Laminin (Fn/Coll IV/Lam);

Fibronectin/Collagen IV (Fn/Coll IV), Collagen IV/Laminin (Collagen IV/Laminin (Coll IV/Lam); Fibronectin/Laminin (Fn/Lam); Collagen IV (Coll IV); Laminin (Lam); Fibronectin (Fn). The final concentration of each ECM composition was 50 μg/ml. The effect of ECM composition on Clara cell behaviour (proliferation, differentiation, death and expression of cyclin kinase inhibitors (CKI) was studied.

The main objectives of this chapter are:

1. To find out whether cell-matrix interaction is important in the regulation of Clara cell proliferation, differentiation and death both in the presence and absence of p21.
2. To describe the functional roles of p21 in Clara cell cultures.
3. To find out whether p21 is involved in cell-matrix interactions.

5.2 Effect of ECM variation on Clara cell differentiation in wt and p21 ko mice.

Cytokeratins are a good marker of epithelial cells differentiation (Gunning et al., 1992; McBride et al., 1999). In this study three cytokeratins 8, 18 and 19 were studied all of which are present in lung epithelial cells including Clara cells. The variation in the degree of cytokeratin expression gives a good indication on the degree of differentiation from one cell type to another to an intermediate cell.

When cells were freshly isolated, thus unattached at day 0, cytokeratins 8, 18 and 19 expressions were found to be lower ($p < 0.05$) in p21 ko mice when compared to wt mice. (Figures 5.1 & 5.2).

In p21 ko mice, the cytokeratins 8, 18 and 19 expressions at time 0 when cell are freshly isolated and not attached to ECM, were significantly lower ($p < 0.05$) when compared to attached cells at time 24, 72 and 120 hours.

In wt mice, the ECM composition did not influence significantly the cytokeratins 8, 18 and 19 expression and the expression of cytokeratins 8, 18 and 19 did not change significantly over the time in culture.

When Laminin was part of the ECM composition in cultured Clara cells from p21 ko mice, cytokeratins 8 and 19 expressions were significantly higher ($p < 0.05$) when compared with ECM without laminin.

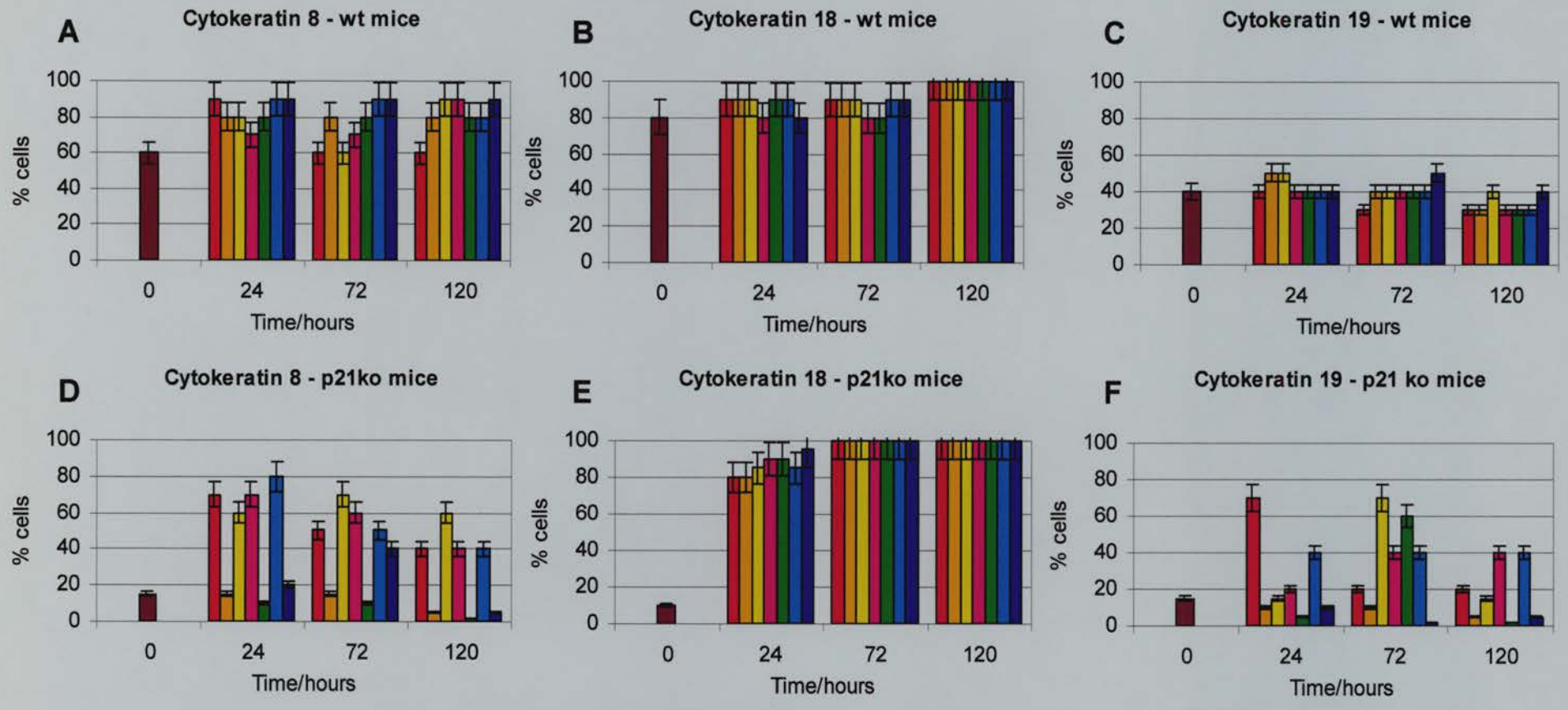

Figure 5.1 - Variation in the expression of cytokeratins 8, 18 and 19 in Clara cells at time 0, 24, 72 and 120 hours in both wt and p21 ko mice in different matrix conditions. At time 0 hours, the expression of cytokeratin 8, 18 and 19 is lower in Clara cells from p21 ko mice compared to cells from wt mice (A – F). The expressions of cytokeratin 8 and 19 in Clara cells from p21 ko mice were higher when laminin part of the ECM composition (D & F).

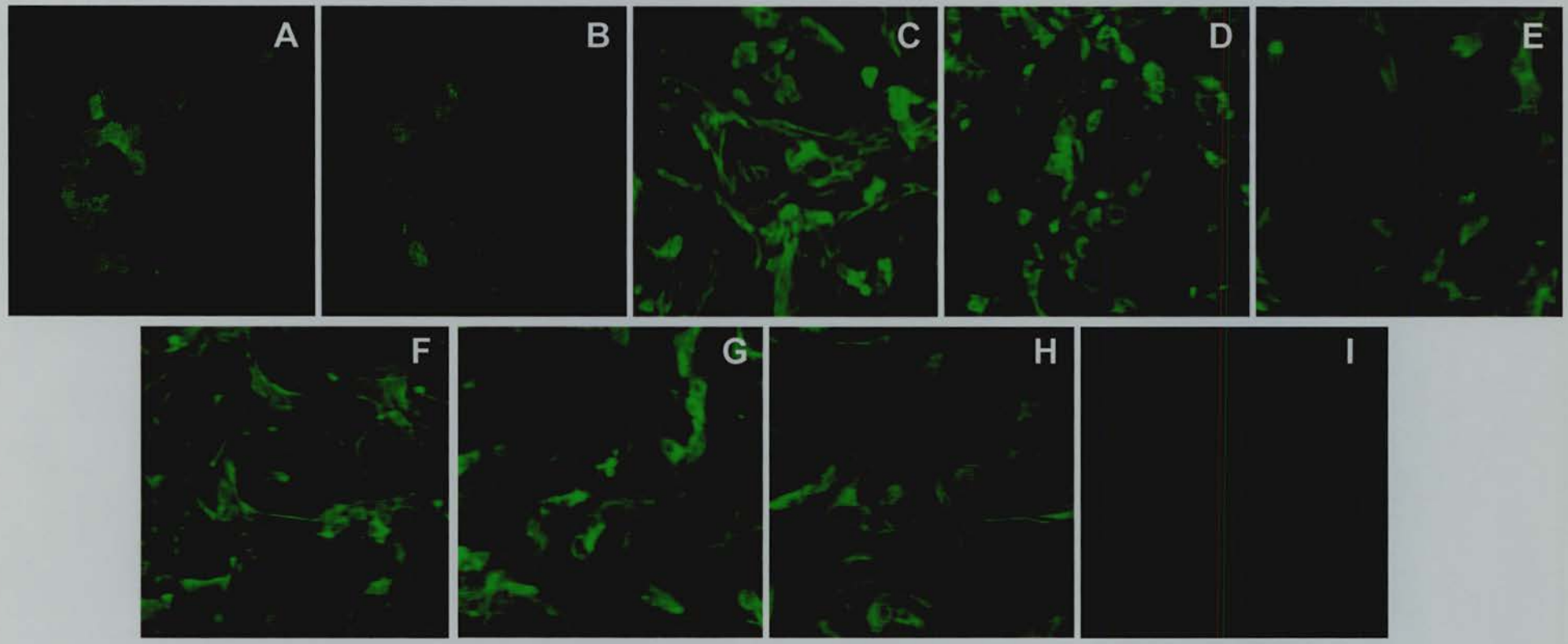

Figure 5.2 - Cytokeratins 8, 18 and 19 expression in different matrix conditions. Cytokeratin 19 expression were found to be lower in Clara cells from p21 ko mice (B) compared to cell from wt mice (A) at time 0 hours. No differences in the expression of cytokeratin 18 was found when cells were cultured on collagen IV (C) and laminin (D). The expression of cytokeratin 8 was found to be higher when cells from p21 ko mice were cultured on ECM containing laminin eg collagen IV/laminin (F) compared to an ECM without laminin eg fibronectin/collagen IV (E), while no difference in cytokeratin 19 in wt mice eg collagen IV/laminin (G) and fibronectin/collagen IV (H). C, D, E, F, G and H are at time point 72 hours. I is a typical negative control, whereby the primary antibody was omitted. Magnification x200.

5.3 Effect of ECM variation on Clara cell proliferation in wt mice and p21 ko mice.

Clara cell proliferation was evaluated by three method: BrdU incorporation, mitotic counts and PCNA immunocytochemistry and counts (Figure 5.3, 5.4 & 5.5).

No significant changes in mitosis, and BrdU incorporated positive nuclei of Clara cells isolated from wt mice when compared to p21 ko mice was observed. A decrease ($p < 0.05$) in the cytoplasmic PCNA expression and an increase ($p < 0.05$) in nuclear PCNA expression in Clara cells from p21 ko mice was observed when compared to wt mice.

ECM composition did not significantly affect rates of mitosis, BrdU incorporation, cytoplasmic and nuclear PCNA expression of Clara cells from either wt or p21ko mice.

Mitosis counts were lower ($p < 0.05$) at time 24 hours compared to cultured cells at 72 and 120 hours from wt and p21 ko mice. Cytoplasmic and nuclear PCNA expressions were lower ($p < 0.05$) from freshly isolated cells at time 0 hours compared to cultured cells at 24, 72 and 120 hours from wt mice. Nuclear PCNA expression was lower ($p < 0.05$) in freshly isolated cells at time 0 hours compared to the cultured cells from p21 ko mice. No change of BrdU incorporation was noted over the time in culture.

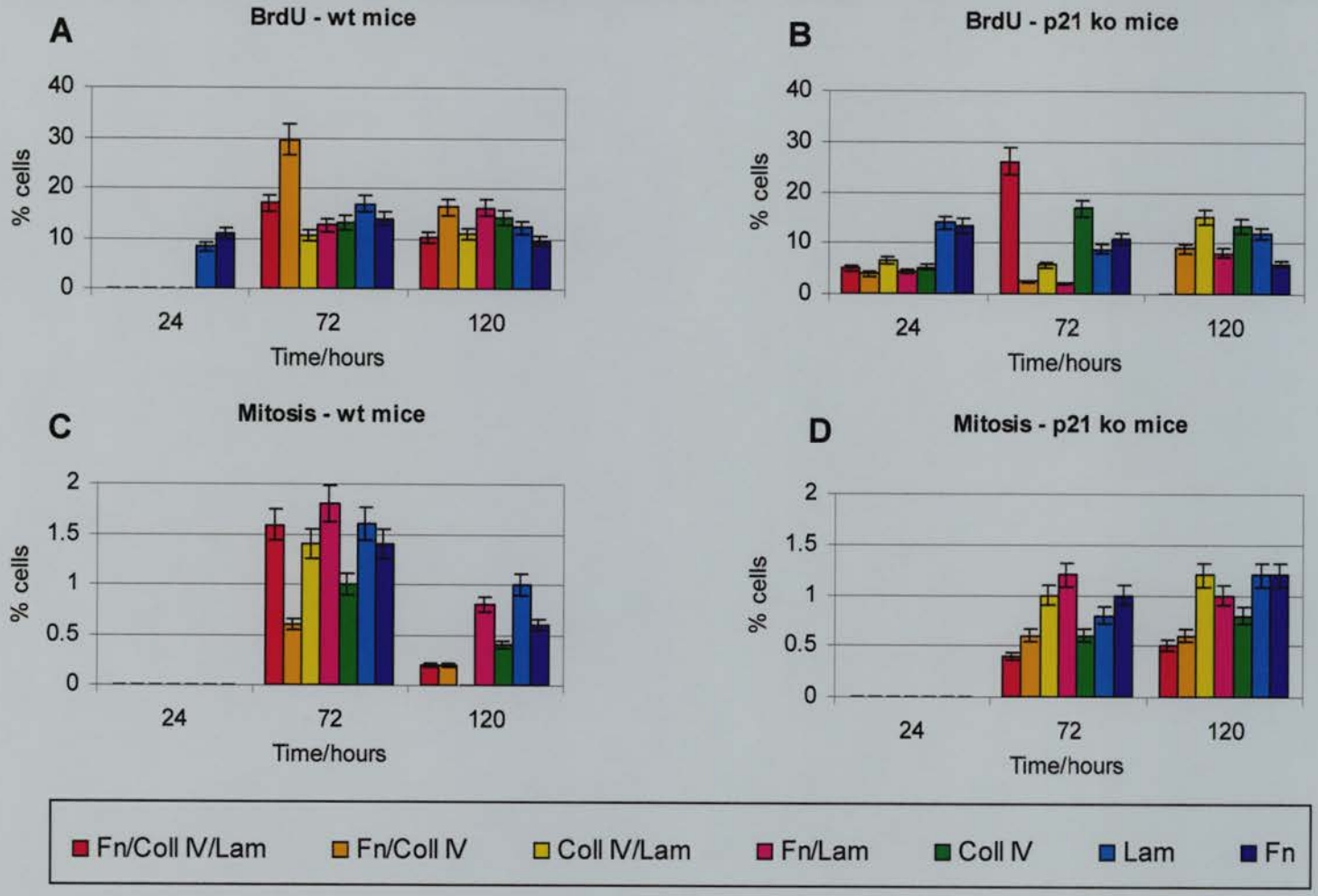

Figure 5.3 - BrdU incorporation and mitosis counts in Clara cells from wt and p21 ko mice in different matrix conditions. No significant changes in the BrdU incorporation and mitosis counts were observed in cells from wt mice when compared to p21 ko mice. ECM did not change significantly BrdU incorporation and mitosis in cells from both wt and p21 ko mice. Mitosis counts at time 24 hours were lower ($p < 0.05$) than at times 72 and 120 hours in cells from both wt and p21 ko mice.

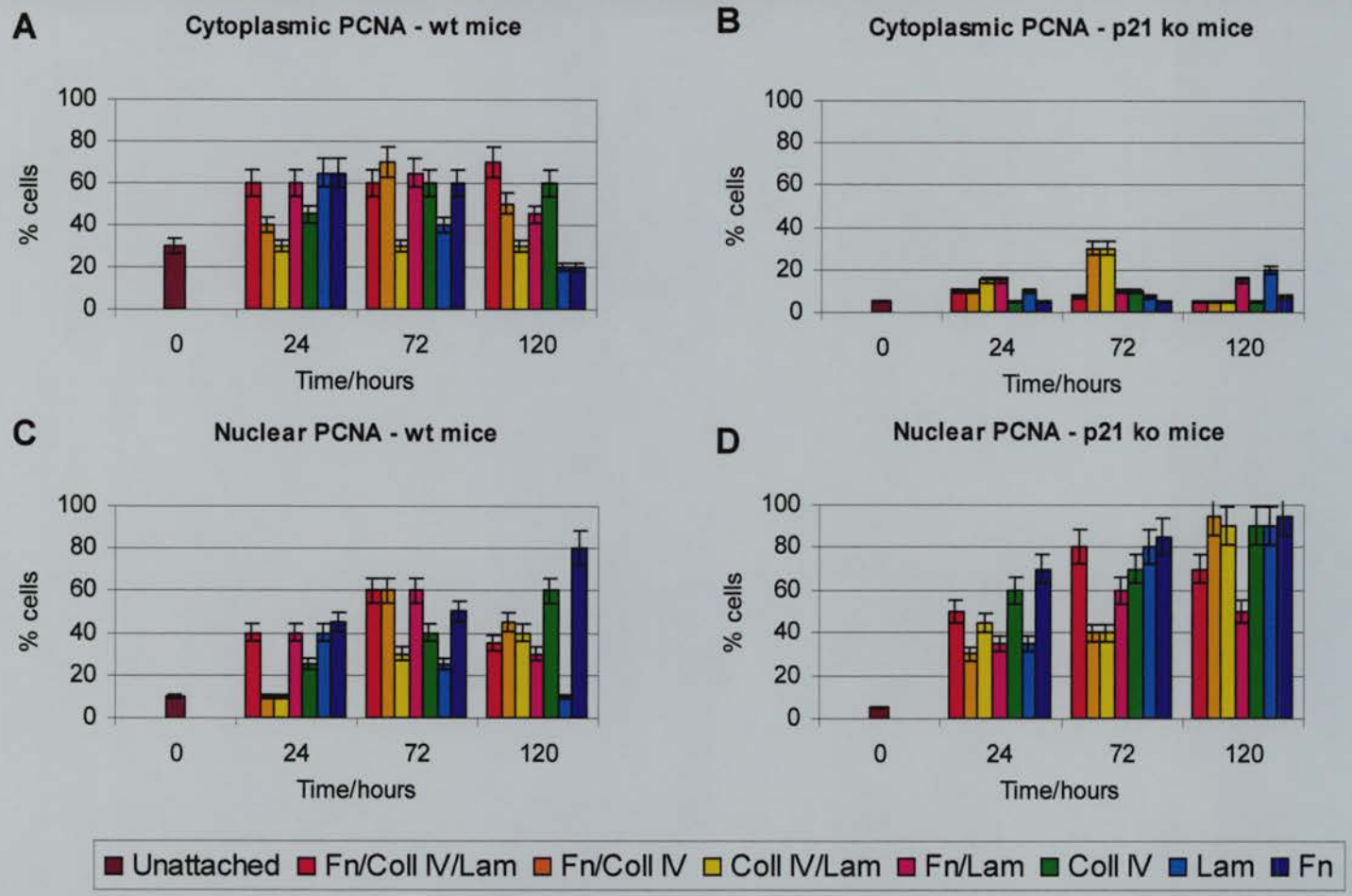

Figure 5.4 - Cytoplasmic and nuclear PCNA expression in Clara cells from wt and p21 ko mice in different matrix conditions. Cytoplasmic PCNA expression was lower ($p < 0.05$) (A & B) but nuclear PCNA expression higher ($p < 0.05$) (C & D) in cells from p21 ko mice when compared to wt mice. ECM did not change significantly PCNA expression in cells from both wt and p21 ko mice. In cells from wt mice, the nuclear and cytoplasmic PCNA expression of freshly isolated cells at time 0 hours was lower ($p < 0.05$) than cultured cells (A & C), while in cells from p21 ko mice, the nuclear PCNA expression was lower ($p < 0.05$) at time 0 hours (D) compared to the other time points.

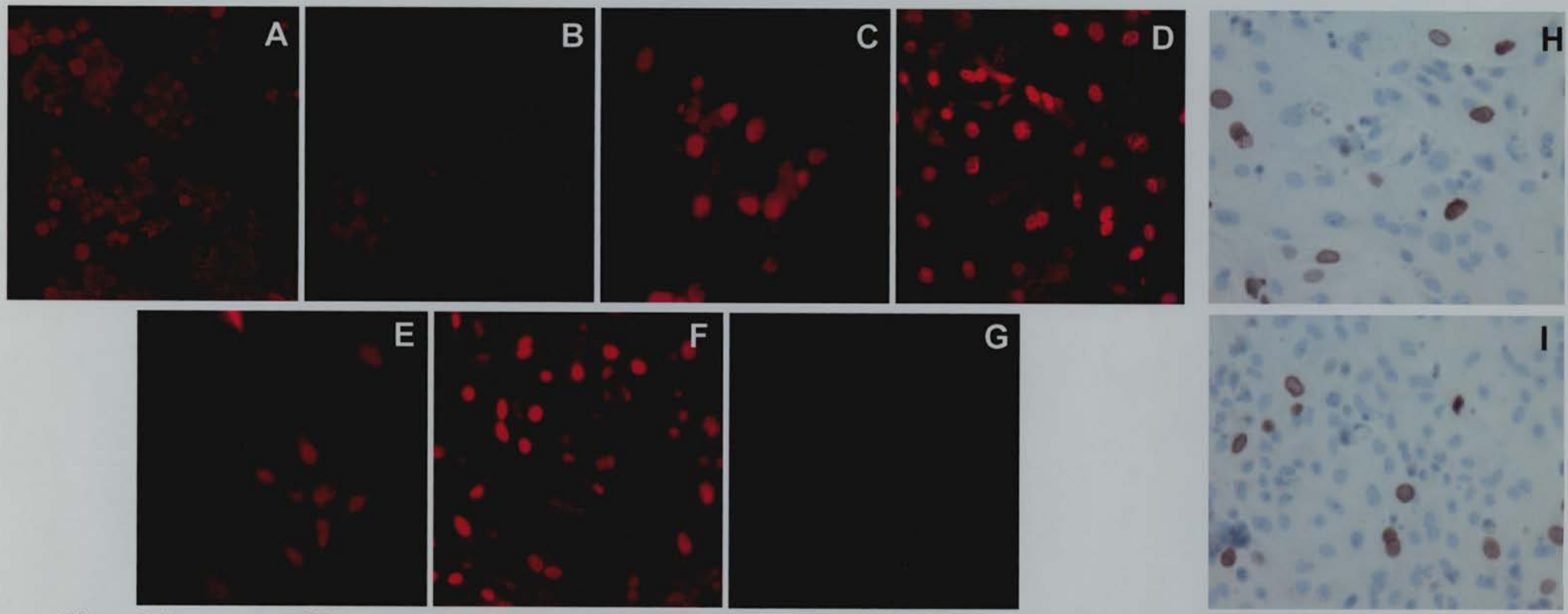

Figure 5.5 - PCNA expression and BrdU incorporation in Clara cell cultures in different matrix conditions. Cytoplasmic PCNA expression was lower ($p < 0.05$) but nuclear PCNA expression higher ($p < 0.05$) in cells from p21 ko mice (D & F) when compared to wt mice (C & E) . ECM did not change significantly PCNA expression in cell from both wt (C & E) and p21 ko mice (D & F). In cells from wt mice, the nuclear and cytoplasmic PCNA expression of freshly isolated cells at time 0 hours (A) were lower ($p < 0.05$) than cultured cells (C & E). In cells from p21 ko mice at time 0 hours (B), the nuclear PCNA expression was lower ($p < 0.05$) compared to cultured cells (D & F). G is a negative control for immunofluorescence whereby the primary antibody was omitted. No changes in the number of BrdU positive nuclei were observed between cells from wt (H) and p21 ko mice (I). Cells in C, D, E, F, G, H & I are at time points 72 hours. Cell from C & D were cultured on Fibronectin/Collagen IV/Laminin while cells in E & F were cultured on Fibronectin/Collagen IV. Magnification x 200.

5.4 Effect of ECM variation on Clara cell death in wt and p21 ko mice.

The necrotic and apoptotic dead cells were counted in primary Clara cell cultures at times 24, 72 and 120 hours respectively using Feulgen staining technique and morphological assessment (Figures 5.6).

The necrotic rates at time points 72 and 120 hours in Clara cells from wt mice were significantly higher ($p < 0.05$) when compared to cells from p21 ko mice. The necrotic rate in Clara cell cultures at 24 hours from wt mice were significantly lower ($p < 0.05$) than cells cultured at time points 72 and 120 hours. The apoptotic rate in Clara cells from wt mice at time point 24 hours was significantly lower ($p < 0.05$) than cells at 72 hours in culture. The apoptotic rate at 72 hours was significantly higher ($p < 0.05$) in cells from wt mice compared to cells from p21 ko mice.

No significant differences in apoptosis or necrosis were observed when Clara cells from both wt and p21 ko mice when cultured in different ECM.

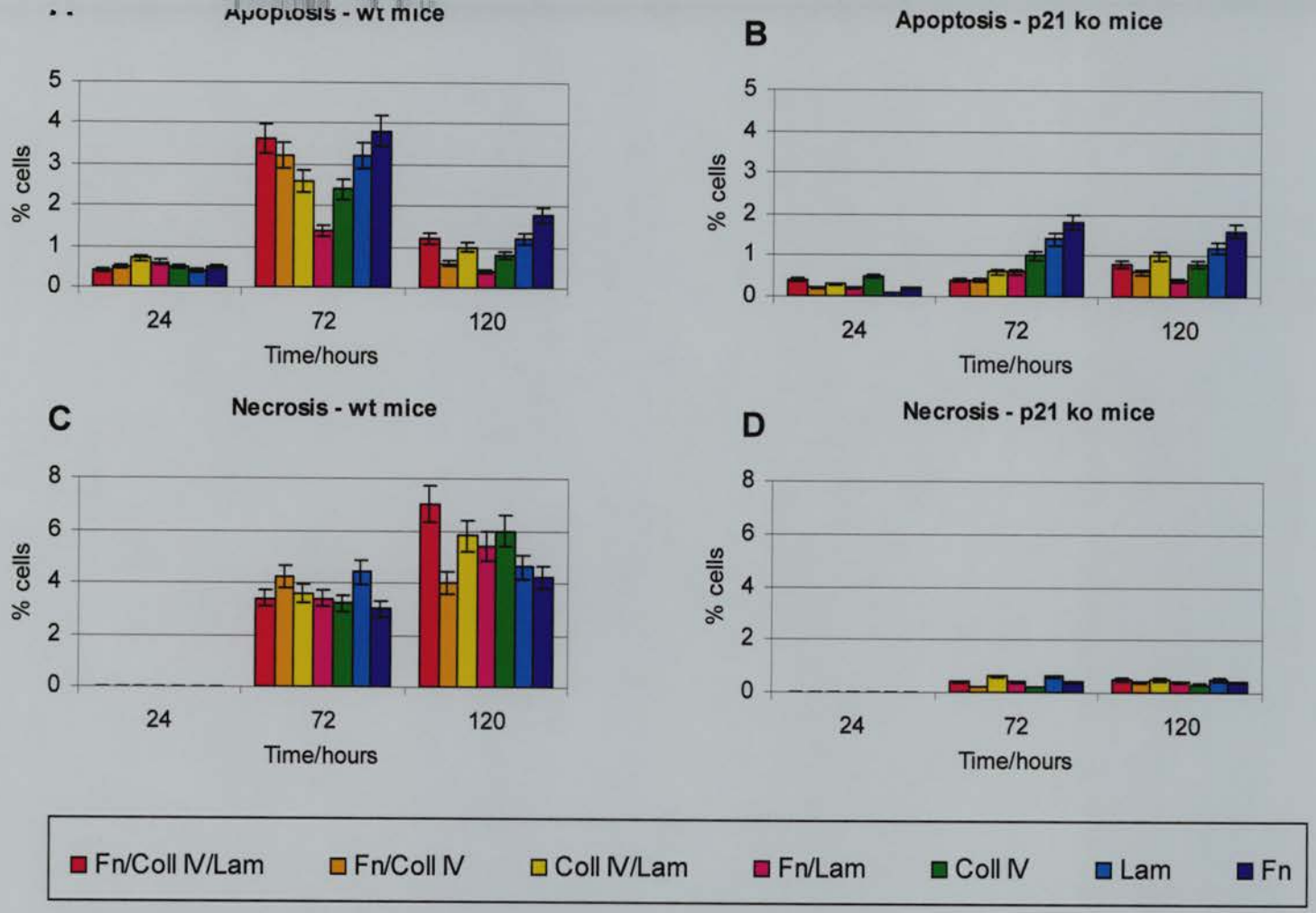

Figure 5.6 - Apoptotic and necrotic rate in Clara cell cultures from wt and p21 ko mice. The apoptosis rate in cells from wt mice (A) was higher ($p < 0.05$) at time point 72 hours compared to cells from p21 ko mice (B). The necrotic rates in cells from wt mice at time points 72 and 120 hours (C) were higher ($p < 0.05$) than cells from p21ko mice (D). The necrotic rate at time 24 hours was lower ($p < 0.05$) when compared to other time points in Clara cells from wt mice (C). No significant differences in apoptosis and necrosis were observed when cells were cultured on different ECM compositions from both wt and p21 ko mice.

5.5 Effect of ECM variation on the expression of cyclin kinase inhibitors p21, p27 and p53 in Clara cells from wt mice and p21 ko mice.

Three cyclin kinase inhibitors p21, p27 and p53 were studied by immunocytochemistry (Figures 5.7, 5.8, 5.9, 5.10).

Nuclear p21 expression increased ($p < 0.05$) from time 0 to 120 hours in culture. Cytoplasmic p21 expression was higher ($p < 0.05$) at time 0 when compared to time 24 hours.

The expression of cytoplasmic p53 was lower ($p < 0.05$) whereas the expression of nuclear p53 was higher ($p < 0.05$) in Clara cells from p21 ko mice compared to cells from wt mice. The expression of cytoplasmic p53 is increasing ($p < 0.05$) from culture times 24 to 120 hours in cells from wt mice. The expression of nuclear p53 is increasing ($p < 0.05$) from time 0 to time 120 hours in culture in cells from p21 ko mice.

The expression of cytoplasmic p27 was found to be higher ($p < 0.05$) in cells from p21 ko mice compared to cells from wt mice at 24 hours in culture. No significant differences in the expression of nuclear p27 were observed between cells from wt and p21 ko mice.

No significant differences in the expression of p21, p27 and p53 were observed when Clara cells were cultured on different ECM compositions.

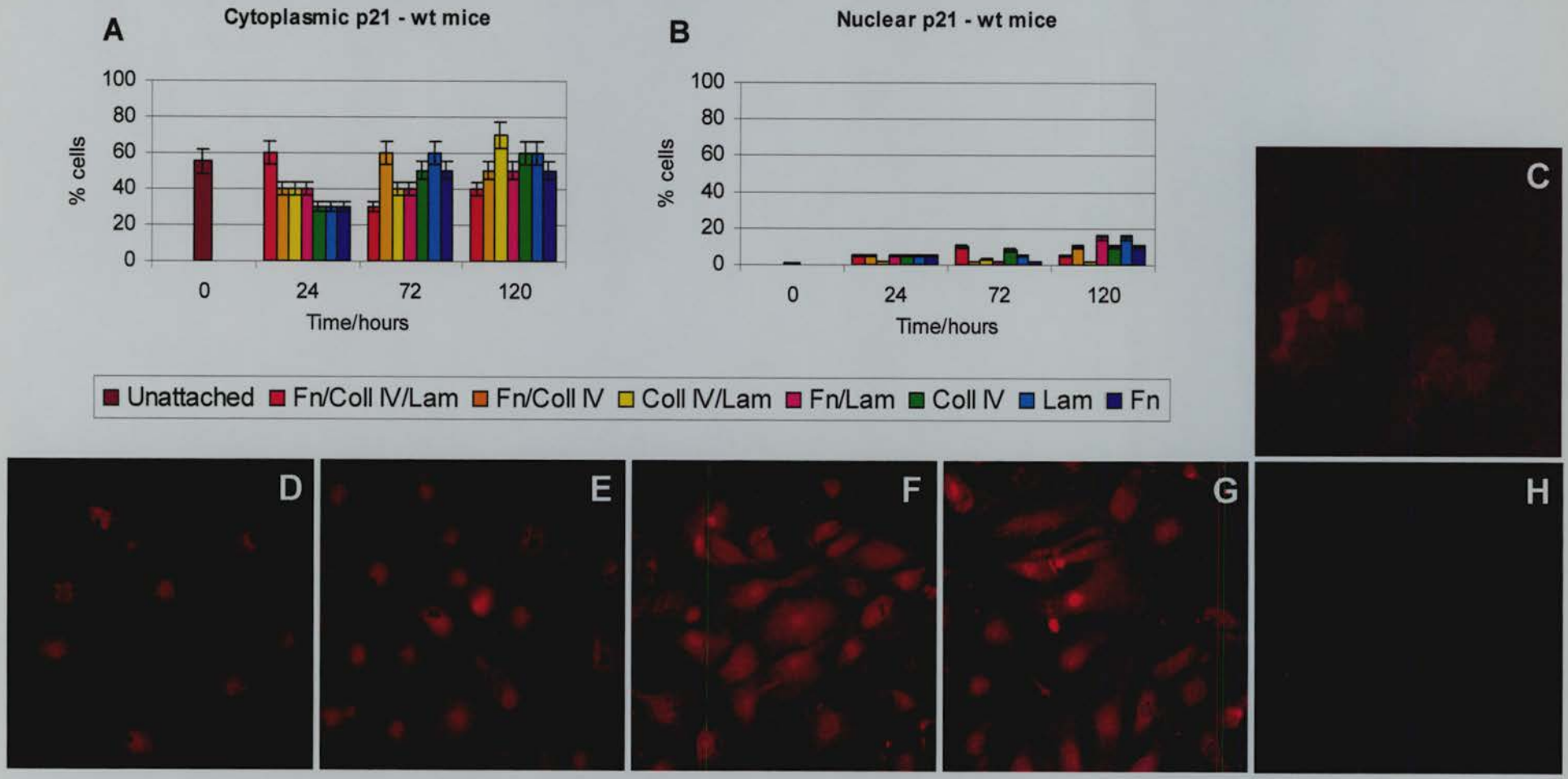

Figure 5.7 - Nuclear and cytoplasmic p21 expression in Clara cell cultures in different matrix conditions. Nuclear p21 expression increased ($p < 0.05$) from time 0 to 120 hours in culture [Graph B, cells at time 0 (C), 24 hours (D), 72 hours (E), 120 hours (F & G). Cytoplasmic p21 expression was higher ($p < 0.05$) at time 0 (C) when compared to time 24 hours (A & D). No significant difference in p21 expression was observed when cells were cultured on different ECMs eg laminin (F) and fibronectin/laminin (G). Cells in D & E were cultured on laminin. H is the negative control whereby the primary antibody was omitted. Magnification x 200.

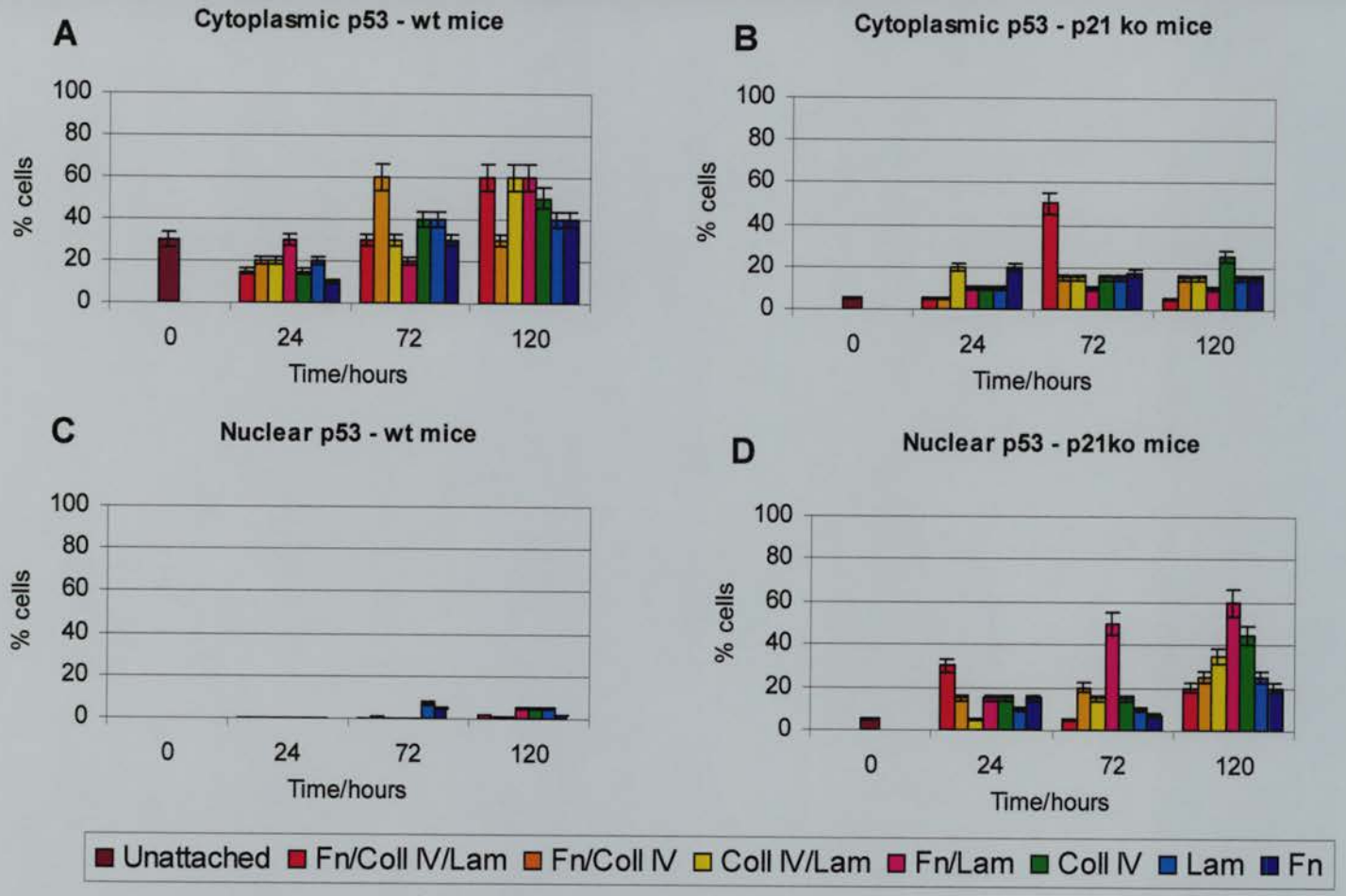

Figure 5.8 - Cytoplasmic and nuclear p53 expression in Clara cells from wt and p21 ko mice in different matrix conditions. The expression of cytoplasmic p53 was lower ($p < 0.05$) (A & B) whereas the expression of nuclear p53 was higher ($p < 0.05$) (C & D) in Clara cells from p21 ko mice compared to cells from wt mice. The expression of cytoplasmic p53 is increasing ($p < 0.05$) from culture times 24 to 120 hours in cells from wt mice (A). The expression of nuclear p53 is increasing ($p < 0.05$) from time 0 to time 120 hours in culture in cells from p21 ko mice (D). The expression of nuclear and cytoplasmic p53 expression did not significantly when cells were cultured on different ECMs (A – D).

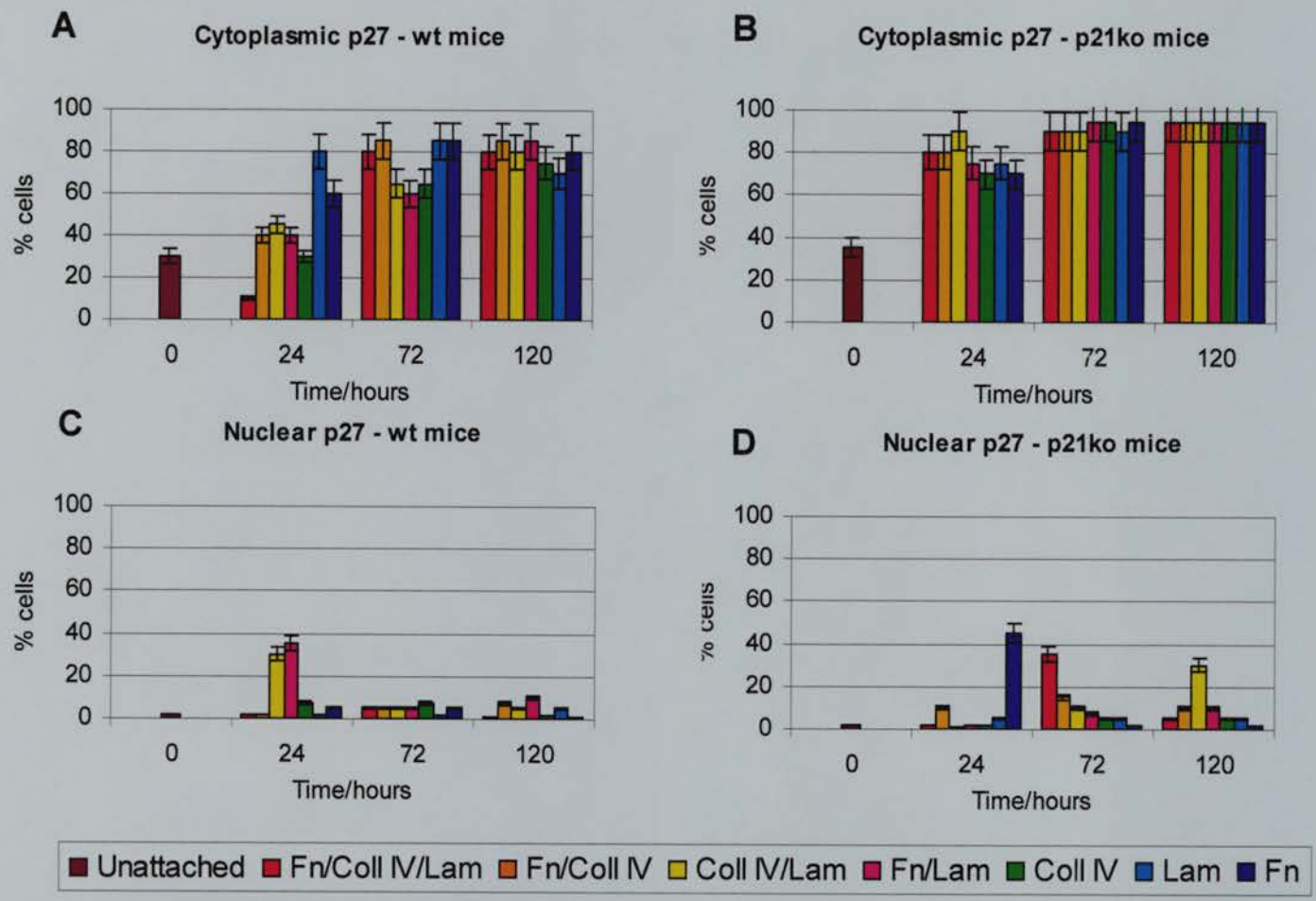

Figure 5.9 - Cytoplasmic and nuclear p27 expression in Clara cells from wt and p21 ko mice in different matrix conditions. The expression of cytoplasmic p27 was found to be higher ($p < 0.05$) in cells from p21 ko mice (A) compared to cells from wt mice (B) at 24 hours in culture. No significant differences in the expression of nuclear p27 were observed between cells from wt and p21 ko mice (C & D). No significant differences in the expression of nuclear and cytoplasmic p27 were observed when Clara cells were cultured on different ECMs (A – D).

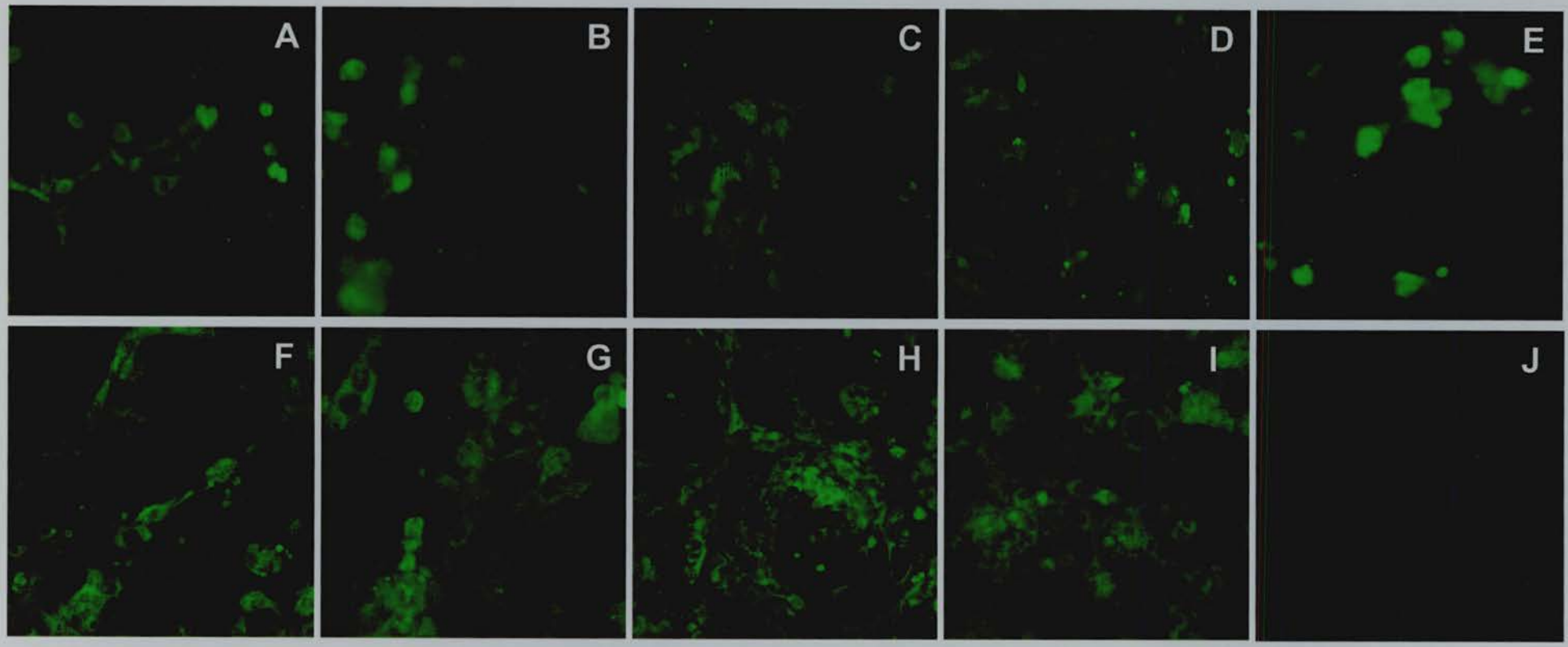

Figure 5.10 - p27 and p53 expression in Clara cells from wt and p21 ko mice in different matrix conditions. The expression of cytoplasmic p53 was lower ($p < 0.05$) whereas the expression of nuclear p53 was higher ($p < 0.05$) in Clara cells from p21 ko mice (B) compared to cells from wt mice (A). The expression of cytoplasmic p53 is increasing ($p < 0.05$) from culture times 24 hrs (A) to 72 hrs (C) to 120 hrs (D) in cells from wt mice. The expression of nuclear p53 is increasing ($p < 0.05$) from 24 hrs (B) to 120 hrs (E) in culture in cells from p21 ko mice. The expression of cytoplasmic p27 was found to be higher ($p < 0.05$) in cells from p21 ko mice (G) compared to cells from wt mice (F) at 24 hours in culture. No significant differences in the expression of nuclear p27 were observed between cells from wt (H) and p21 ko mice (I) at 120 hours in culture. J is a negative control whereby the primary antibody was omitted. Cells in A – E were cultured on Fibronectin/Collagen IV/Laminin while cells in F – J were cultured on Collagen IV. Magnification x 200.

5.6 Discussion.

5.6.1 Lower expression of cytokeratins 8, 18 and 19 in unattached Clara cells from p21 ko mice compared to wt mice.

Differences in cytokeratin expression in lung cells have been established and have been used as differentiation markers in lung epithelial cells. (Gunning et al., 1992; Moll et al., 1982; Moll, 1987; Blobel et al., 1984). The expression of cytokeratin 8, 18 and 19 in freshly isolated Clara cells from p21 ko mice was significantly lower ($p < 0.05$) than cultured cells. By disrupting the cell-matrix interactions, in this case during the isolation procedure, the unattached Clara cells from p21 ko mice could be undergoing differentiation and thus cytokeratins 8, 18 and 19 expression was lower.

No significant changes in the expression of cytokeratin 8, 18 or 19 was observed in Clara cells from wt mice upon the cell-matrix disruption and when cultured on different ECM. Thus changes in cell-matrix interactions could be an important factor for cells to undergo differentiation through a p21-dependent pathway. In p21 ko mice, normal differentiation has been observed, thus p21 is not a mutually exclusive agent that promotes differentiation (Paramio et al., 2001; Cox, 1997; McDonald et al., 1996; Deng et al., 1995).

When Clara cells from p21 ko mice were attached and cultured to an ECM which contained laminin, the expression of cytokeratin 8 and 19 was significantly higher ($p < 0.05$) than cells cultured in the absence of laminin. Thus in the absence of p21, the presence of laminin was found to be quite important for cells to undergo normal differentiation. The actual pathway by which the presence of laminin and p21 are involved in differentiation in Clara cells is still unclear and further studies need to be carried out.

5.6.2 ECM composition does not influence Clara cells proliferation both in the presence and absence of p21.

No significant changes in the proliferation rate were observed in Clara cells from wt mice as compared to cells from p21 ko mice. Thus under these serum-free cell culture conditions, p21 does not affect Clara cell proliferation. Although p21 was shown to inhibit proliferation (Balomenos et al., 2000; Sugibayashi et al., 2002; Harper et al., 1993; El-Deiry et al., 1993; Harper et al., 1995; Niculescu et al., 1998), normal development and proliferation was also described in mice lacking p21 (Brugarolas et al., 1995; Deng et al., 1995; Waldman et al., 1996; McDonald et al., 1996; Weinberg et al., 1997; Alan Wang et al., 1997). The role of p21 regarding proliferation is still unclear and this could be due to different cell types and also due to different culture conditions. A significant increase in proliferation in Clara cells from p21 ko mice was observed as compared cells from wt mice, when serum was used in the culture medium (unpublished results).

Although culturing Clara cells on different ECM composition did not affect the proliferation rate, the mitotic rate was lower ($p < 0.05$) at culture time 24 hours compared to cultured cells at 72 and 120 hours from both wt and p21 ko mice. This could be due that cells at 24 hours in culture are not as attached to the ECM as cells at 72 and 120 hours in culture. Thus proliferation in Clara cells is anchorage dependent although not directly influenced by ECM composition.

A significant ($p < 0.05$) decrease in cytoplasmic PCNA and an increase ($p < 0.05$) in nuclear PCNA expression was observed in Clara cells from p21 ko mice as compared to wt mice. PCNA is required for both DNA replication and DNA repair (Li et al., 1995a; Moriuchi, 1990; Oku et al., 1998; Rossi et al., 1999; Rousseau et al., 1999; Yu et al., 2001). Since no significant changes in proliferation were observed using BrdU incorporation and mitotic counts, these changes in the PCNA expression could be attributed to repair.

Nuclear PCNA expression was lower in the freshly isolated cells at time 0 compared to cultured cells from both wt and p21 ko mice, while cytoplasmic PCNA expression was lower only in cells from wt mice. Thus it can be hypothesised that the cell-matrix disruption increases the nuclear PCNA expression in Clara cells from both wt and p21 ko, which is most probably involved in DNA repair. Most probably p21 has a role in controlling transportation of PCNA to the nucleus, since a decrease ($p < 0.05$) in cytoplasmic PCNA expression but an increase ($p < 0.05$) in nuclear PCNA expression was observed in Clara cells from p21 ko mice when compared to cells from wt mice. The role of p21 in transportation of PCNA to the nucleus has been reported (Li et al., 1994; Li et al., 1995a; Funk et al., 1997; Warbrick et al., 1997; Cayrol et al., 1998; Oku et al., 1998; Rousseau et al., 1999).

5.6.3 p21 can induce apoptosis and necrosis in Clara cells.

Necrotic rate of Clara cells from wt mice at 72 and 120 hours in culture were significantly higher ($p < 0.05$) than cell from p21 ko mice. Apoptotic rate was higher ($p < 0.05$) in Clara cells from wt mice at 72 hours in culture compared to cells from p21 ko mice. Thus p21 seems to be involved in promoting necrosis and apoptosis death pathways.

There were no significant changes in the apoptosis and necrosis rates by culturing Clara cells on different ECM. A number of studies has shown that when cells are detached from substrate or anchorage is prevented they usually undergo apoptosis (Ruoslahti and Reed, 1994; Frisch and Ruoslahti, 1997; Frisch and Francis, 1994; Schwartz, 1997; Assoian, 1997; Day et al., 1997; Meredith et al., 1993; McGill et al., 1997; Ilic et al., 1998; Vitale et al., 1999; Kettritz et al., 1999; Sethi et al., 1999). The necrotic and apoptotic rates were lower ($p < 0.05$) at culture time 24 hours compared to cultured Clara cells at 72 hours from wt mice. Cells at 24 hours in culture are most probably less attached to the ECM compared to cells at 72 and 120 hours in culture, thus some cells could have been detached and not detected in the

counts thereby explaining the unexpected low death at 24 hours in culture. On the other hand, due to the fact that Clara cells were cultured in a serum free condition, there could be some growth factors missing in the medium and so as culture time in culture increases, the cell death rate also increases. Most probably both statements are correct and the truth somewhere in between. Further studies need to be carried out to find out whether disruption of cell-matrix interaction leads to apoptosis via a p21-dependent pathway.

5.6.4 Decrease in cytoplasmic p21 upon adherence.

In this study an increase ($p < 0.05$) in the nuclear p21 expression was observed from time 0 to 120 hours in culture. Cytoplasmic p21 expression was higher in unattached Clara cells at time 0 compared to cells at 24 hours in culture thus cytoplasmic p21 expression could be increased upon cell-matrix disruption. The functional role of cytoplasmic p21 is still unclear (Asada et al., 1999; Tchou et al., 1996; Poon and Hunter, 1998; Donato and Perez, 1998; Zhang et al., 1999; Jin et al., 2000; Gervais et al., 2000), thus further studies need to be carried out to understand its functional role. Culturing different Clara cells on a variation of ECM combinations did not significantly affect the expression of p21 thus cell-matrix disruption rather than ECM composition seems to be more important.

5.6.5 Nuclear p53 expression increases in Clara cells lacking p21.

The expression of cytoplasmic p53 was lower whereas the expression of nuclear p53 was higher ($p < 0.05$) in Clara cells from p21 ko mice as compared to cells from wt mice. Thus it seems that in the absence of p21, there is an increase in p53 stabilisation. The p53 protein is a potent inhibitor of cell growth, arresting the cell cycle at several points and under some circumstances, activating the apoptotic machinery leading to cell death (Brambilla and Brambilla, 1997; Hupp et al., 2000; Ji

et al., 1997; Jimenez et al., 1999; Kaelin, 1999b; Kamijo et al., 1998; Lakin and Jackson, 1999; Lane, 1992; May and May, 1999; Morgenbesser et al., 1994; Sigal and Rotter, 2000; Stewart et al., 2001; Vaziri and Benchimol, 1999; Vitale et al., 1999; Wyllie et al., 1994; Yap et al., 1999). The role of cytoplasmic p53 is still unclear although cytoplasmic localisation of wildtype p53 has been reported in inflammatory breast carcinoma, colorectal adenocarcinoma, undifferentiated neuroblastoma (Ostermeyer et al., 1996; Moll et al., 1995), hepatocellular carcinoma and retinoblastoma (Morgenbesser et al., 1994). Culturing different Clara cells on a variation of ECM combinations did not affect the expression of p53.

5.6.6 Increase in the cytoplasmic p27 in Clara cells lacking p21 at 24 hours in culture.

The expression of cytoplasmic p27 was found to be higher ($p < 0.05$) in Clara cells from p21 ko mice compared to cells from wt mice at 24 hours in culture, but no significant differences in the expression of nuclear p27 were observed in cells from wt and p21 ko mice at any time point in culture. Nuclear p27 negatively regulates G1 progression by binding to cyclin D-cdk 4/6 complexes, cyclin A/cdk2 and cyclin E-cdk 2 preventing their activity (Lloyd et al., 1999; Harvat et al., 1998; Cheng et al., 1999; Rodier et al., 2001; Reed et al., 1994), but the functional role of cytoplasmic p27 is still unknown although it is thought to be involved in the activation of Cdk 2 complexes (Orend et al., 1998; Rodier et al., 2001; Levkau et al., 1998a). Thus the absence of p21 could lead to an increase in cytoplasmic p27 upon cell attachment to ECM and therefore inhibiting G1 progression and maybe lead to cell differentiation. Cytoplasmic p27 was shown previously to be able to inhibit G1 progression (Orend et al., 1998; Rodier et al., 2001; Tomoda et al., 1999; Tomoda et al., 1999; Reynisdottir and Massague, 1997; Soucek et al., 1998; Lloyd et al., 1999; Nakayama et al., 1996; Ishida et al., 2000; Resnitzky et al., 1995). The p27 expression did not significantly change upon culturing different Clara cells on a variation of ECM combinations, but rather seems to be influenced by the disruption of cell-matrix interactions.

5.6.7 Conclusion.

Although the presence of laminin was found to be important in cytokeratin 8 and 19 expression in Clara cells from p21 ko mice, the cell-matrix disruption rather than ECM combinations seems to have greater influence on cell cycle progression in the absence of p21. It has been previously shown that disruption of cell-matrix interactions affects the expression of p21 (Assoian, 1997; Wu and Schönthal, 1997; Nagaki et al., 2000; Ilic et al., 1998; Bao et al., 2002) but the exact mechanism is still unclear. Specific integrins that bind to laminin rather than different ECM combinations seem to be more important in the regulation of cell cycle progression in Clara cells. Thus, further studies have to be carried out in order to understand the role of specific integrins adhesion or detachment on p21 expression and on cell cycle progression in Clara cell cultures.

Chapter 6 Effect of beta-1 integrin blocking on Clara cell proliferation, differentiation and death through p21 regulation.

6.1 Introduction

Integrins are the primary receptors used by cells to interact with extracellular matrix. Both ECM and integrins play an important part in the survival, proliferation and death of cells. When an injury occurs in lung epithelial cells signals through the integrins could significantly effect whether disease or repair occur.

As observed from chapter 5, cell-matrix disruption rather than ECM compositions itself seems to influence cell cycle progression. When primary Clara cells were cultured on an ECM composition where Laminin was present, differences in the cell cycle progression in wt mice could be seen when compared to p21 ko mice.

Laminin attaches to a number of integrins including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$ and $\alpha_6\beta_4$ (Belkin and Stepp, 2000; Sannes et al., 1998; Vivinus-Nebot et al., 1999; Klinowsks et al., 1999; Burkin et al., 2000; Pance et al., 2001; Hynes, 1992; Kumar, 1998; Damsky and Werb, 1992; Fornaro and Languino, 1997; Sheppard, 2000; van der Flier and Sonnenberg, 2001). Various β_1 and β_4 integrin complexes were shown to be involved in the activation of a number of cell cyclin inhibitors (Giancotti, 1997; Pu and Streuli, 2002; Montgomery et al., 1994; Ruoslahti and Reed, 1994; Clarke et al., 1995; Dixiti et al., 1996; Vivinus-Nebot et al., 1999; Miyata et al., 2000; Schwartz and Assoian, 2001).

$\alpha_6\beta_4$ was shown to activate p53 function in carcinoma cells (Bachelder et al., 1999a) and p53 was found to inhibit $\alpha_6\beta_4$ integrin survival signalling (Bachelder et al.,

1999b). The β_4 integrin cytoplasmic domain was shown to be involved in the activation of p21 pathways of growth arrest and apoptosis (Clarke et al., 1995). T cell proliferation in the human thymus was found to be controlled via $\alpha_6\beta_4$ by laminin 5 (Vivinus-Nebot et al., 1999).

$\alpha_5\beta_1$, a fibronectin binding integrin, was found to negatively regulate cell growth in the absence of attachment to fibronectin (Varner et al., 1995). $\alpha_5\beta_1$ was reported to be involved in the expression of tumour suppressor $p16^{INK4a}$ upon loss of anchorage (anoikis) in a number of human cancer cells (Plath et al., 2000) and also supports survival of cells by up-regulating Bcl-2 expression (Zhang et al., 1995). Trypsin was also found to stimulate integrin $\alpha_5\beta_1$ dependent adhesion to fibronectin (Miyata et al., 2000). $\alpha_6\beta_1$ was shown to be an essential step accompanying the neoplastic transformation of hepatocytes (Carloni et al., 1998).

The main hypothesis of this section is that integrins that bind to laminin influence cell cycle progression possible through the regulation of p21. To study this hypothesis, Clara cells from both wt and p21 ko mice were cultured on Laminin at a concentration of 50 μg/ml and incubated with either a β_1 blocking antibody ($HM\beta_1$) or with a cytokeratin 8 antibody as a control. Both antibodies were used at a concentration of 10 μg/ml. The $HM\beta_1$ antibody has been shown previously to react with mouse β_1 and has an inhibitory effect on cell adhesion to ECM proteins (Noto et al., 1995; Burns et al., 2001). The cytokeratin 8 antibody does not have an inhibitory function.

The main objectives of these experiments are:

1. To study the roles of cell-adhesion of β_4 integrin and cell-disruption of β_1 integrin in primary Clara cell cultures..
2. To determine the effect on cell cycle progression upon cell-matrix disruption.
3. To determine the functional role of p21 upon cell-matrix disruption.

6.2 Effects of beta 1 integrin blocking on cytokeratins expression in Clara cells from wt and p21 ko mice.

No significant changes in the expression of cytokeratins 8, 18 and 19 were observed upon beta-1 integrin blocking in cells from wt mice as compared to cells from p21 ko mice at any time point in culture.

The expression of cytokeratin 8 was significantly lower ($p < 0.05$) in the control cells (not beta-1 integrin blocked) from p21 ko mice at time 72 and 120 hours compared to cells from wt mice control and beta-1 blocked cells from p21 ko mice at the same time points.

A significant decrease ($p < 0.05$) in the expression of cytokeratin 18 was observed in cells from wt mice at time 72 hours in culture upon beta-1 integrin blocking.

A significant increase ($p < 0.05$) in the expression of cytokeratin 19 was noted in both cells from wt and p21 ko mice at time 120 hours in culture upon beta-1 integrin blocking.

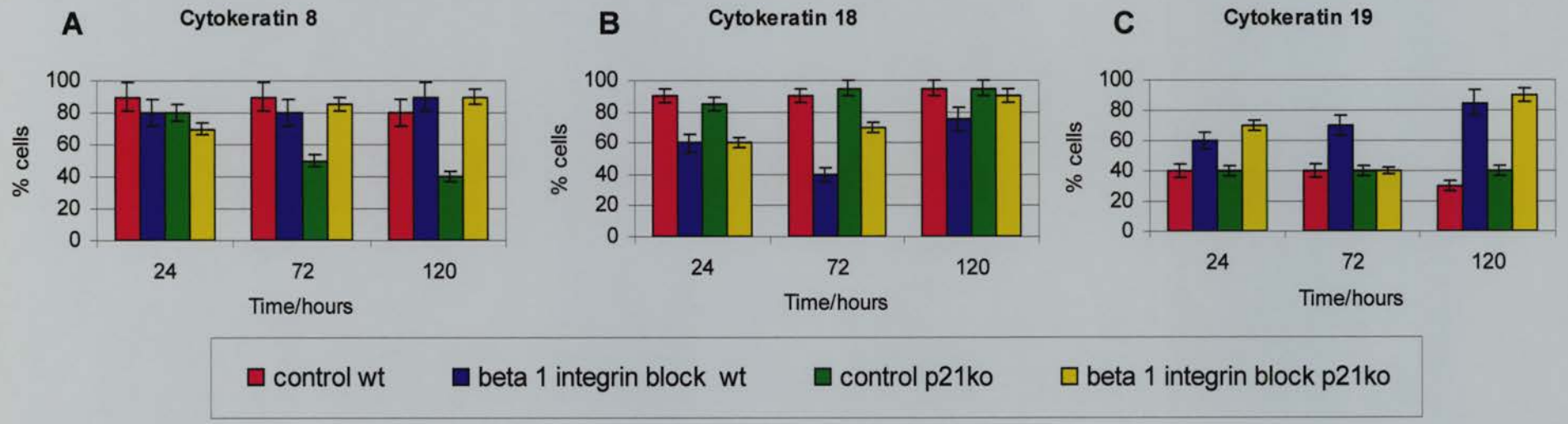

Figure 6.1 - Changes in the expression of cytokeratins 8, 18 and 18 in Clara cells at time 24, 72 and 120 hours in both wt and p21 ko mice upon beta-1 integrin blocking. No significant changes in the expression of cytokeratins 8, 18 and 19 were observed upon beta-1 blocking in cells from wt mice as compared to cells from p21 ko mice (A, B & C). Cytokeratin 8 expression was lower ($p < 0.05$) in control cells from p21 ko mice at culture times 72 and 120 hours (A). Cytokeratin 18 expression was lower ($p < 0.05$) in beta-1 blocked cells from wt mice at culture time 72 hours (B). Cytokeratin 19 expression was higher ($p < 0.05$) in beta-1 blocked cells from both wt and p21 ko mice at culture time 120 hours (C).

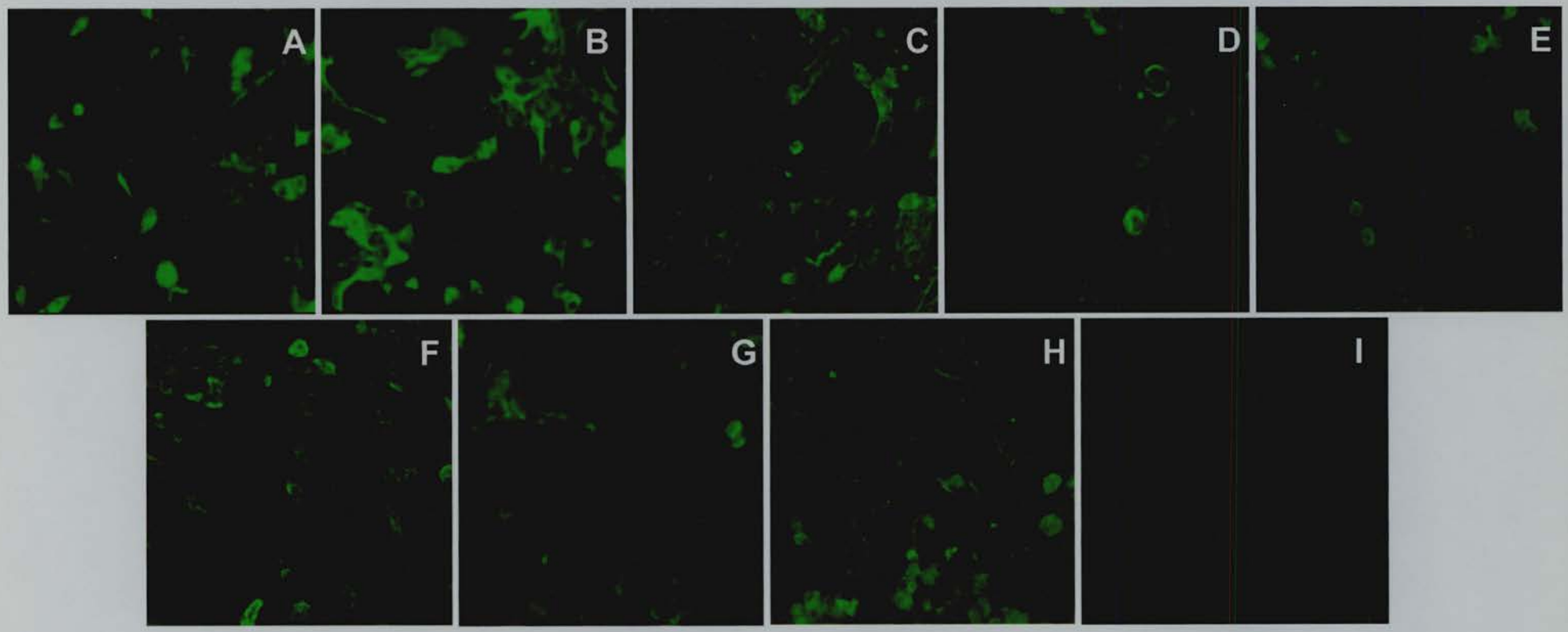

Figure 6.2 - Changes in the expression of cytokeratins 8, 18 and 18 in Clara cells in culture from wt and p21 ko mice upon beta-1 integrin blocking. Cytokeratin 8 expression was lower ($p < 0.05$) in control cells from p21 ko mice (A) at culture times 120 hours compared to beta-1 blocked Clara cells (B). Cytokeratin 18 expression was lower ($p < 0.05$) in beta-1 blocked cells from wt mice at culture time 72 hours (D) compared to control (not beta-1 blocked) wt cells (C). Cytokeratin 19 expression was higher ($p < 0.05$) in beta-1 blocked cells from both wt (F) and p21 ko mice (H) at culture time 120 hours compared to controls [wt (E) & p21 ko (G)]. I is the negative control for immunofluorescence whereby the primary antibody was omitted. Magnification x 200.

6.3 Effects of beta 1 integrin blocking on Clara cell proliferation in wt and p21 ko mice.

Clara cell proliferation was evaluated by three different methods: BrdU incorporation, mitosis count and PCNA immunocytochemistry and counts.

The expression of cytoplasmic PCNA was significantly higher ($p < 0.05$) in cells from wt and p21 ko mice upon beta-1 integrin blocking at time 120 hours in culture as compared to the control (using a non-blocking antibody). The expression of nuclear PCNA increased significantly ($p < 0.05$) in cells from wt mice upon beta-1 integrin blocking at time 72 and 120 hours in culture.

A significant decrease ($p < 0.05$) in BrdU incorporation was observed in cells from wt mice upon beta-1 integrin blocking at times 24 and 72 hours in culture. A decrease ($p < 0.05$) in BrdU incorporation was noticed in cells from p21 ko mice upon beta-1 blocking at 24 hours in culture.

No significant changes in the mitosis were observed both in cells from wt and p21 ko mice upon beta-1 integrin blocking.

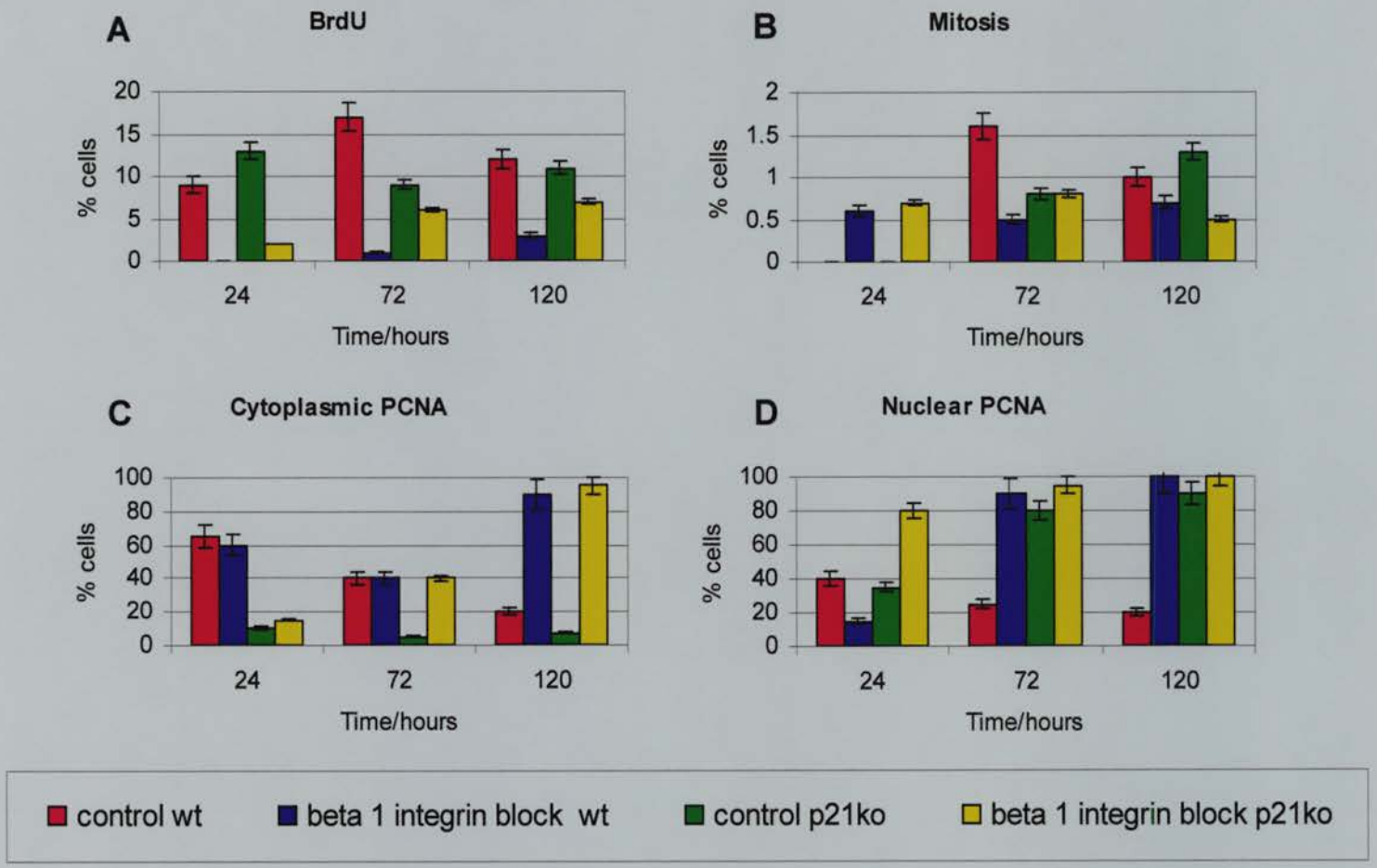

Figure 6.3 - Changes in Clara cell proliferation upon beta-1 integrin blocking in wt and p21 ko mice. A decrease ($p < 0.05$) in BrdU incorporation upon beta-1 blocking was observed in cells from wt mice at 24 and 72 hours in culture, and in cells from p21 ko mice at 24 hours in culture (A). No significant changes in mitosis were observed (B). Cytoplasmic PCNA expression levels increased ($p < 0.05$) in cells from wt and p21 ko mice upon beta-1 blocking at 120 hours in culture (C). Nuclear PCNA expression increased ($p < 0.05$) in cells from wt mice upon beta-1 blocking at 72 and 120 hours in culture (D).

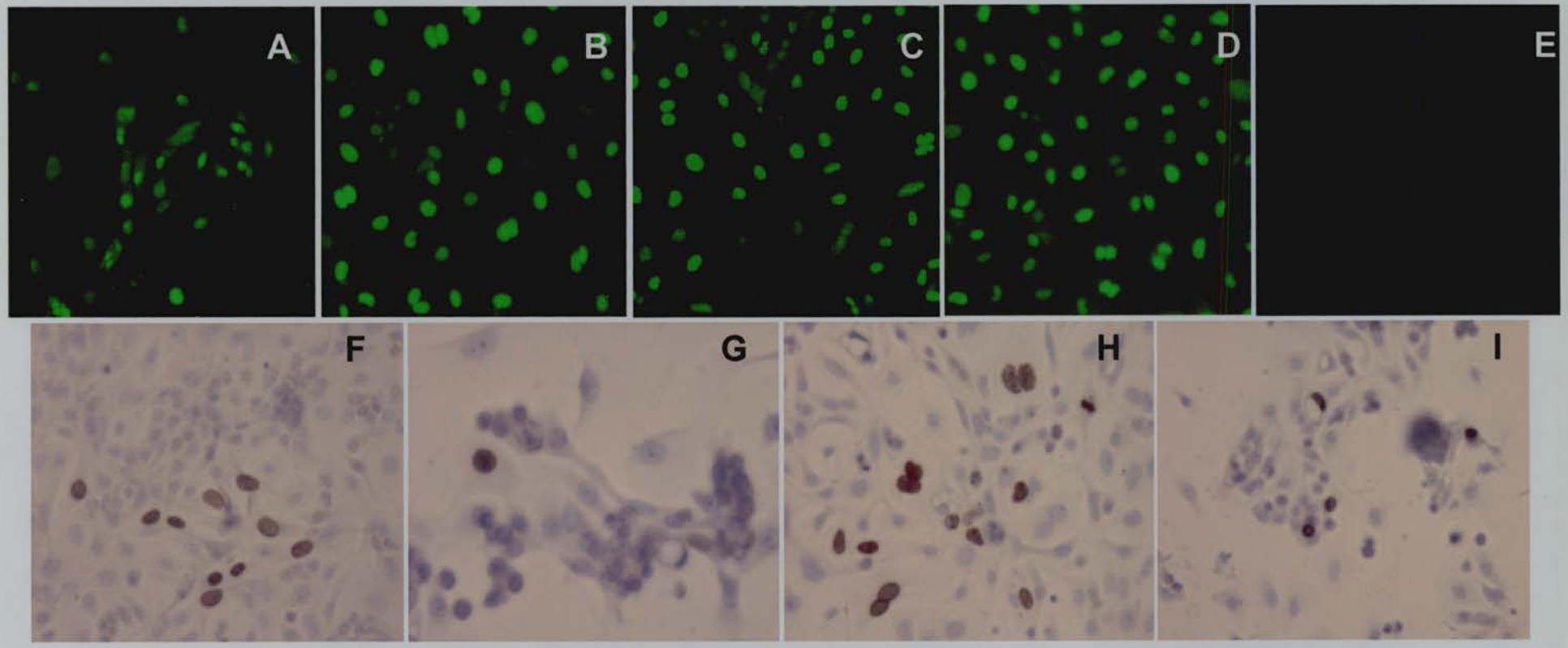

Figure 6.4 - Changes in Clara cell proliferation upon beta-1 integrin blocking in wt and p21 ko mice. Upon beta-1 integrin blocking, the cytoplasmic PCNA expression increased ($p < 0.05$) in Clara cells from wt (B) and p21 ko mice (D) as compared to control cells (not beta-1 blocked) [wt (A) & p21 ko (C)] at 120 hours in culture. Nuclear PCNA expression increased ($p < 0.05$) in cell from wt mice (A) upon beta-1 blocking at 120 hours in culture (B). A decrease ($p < 0.05$) in BrdU incorporation upon beta-1 blocking was observed in cells from wt (G) and p21 ko mice (I) at 24 and 120 hours in culture as compared to control cells [wt (F), p21ko (H)]. E is the negative control for immunofluorescence whereby primary antibody was omitted. Magnification x 200.

6.4 Effect of beta 1 integrin blocking on Clara cell death in Clara cells from wt and p21 ko mice.

Necrotic and apoptotic cells were counted in primary Clara cell cultures at times 24, 72 and 120 hours respectively using Feulgen staining technique (Figure 6.5).

A significant increase ($p < 0.05$) in apoptosis was observed upon beta-1 integrin blocking in Clara cells from wt and p21 ko mice at 72 and 120 hours in culture. Apoptosis was higher ($p < 0.05$) in cells from wt mice compared to cells from p21 ko mice at 72 hours upon beta-1 integrin blocking.

No significant differences in necrosis were observed upon beta-1 integrin blocking in both cells from wt and p21 ko mice.

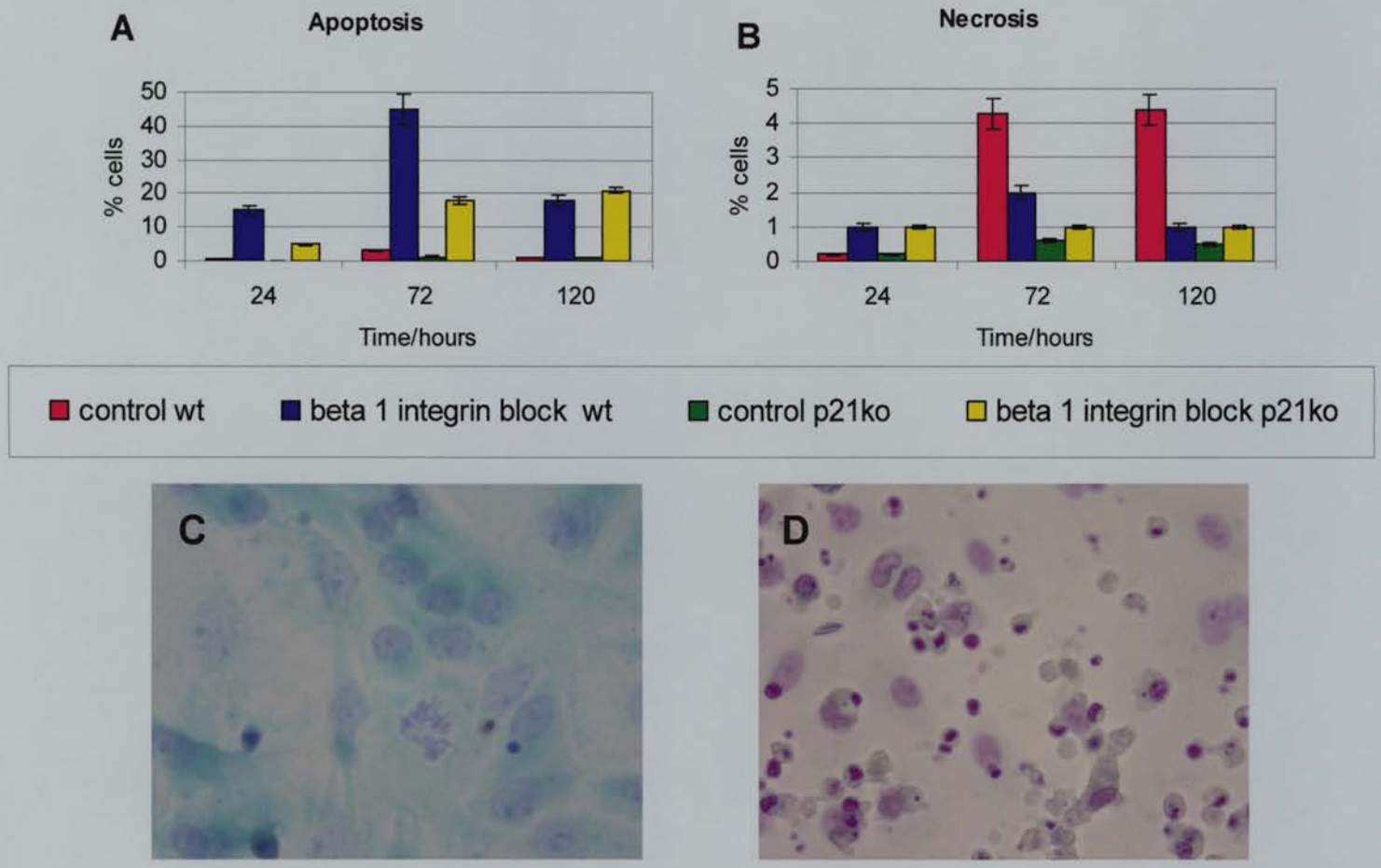

Figure 6.5 - Changes in Clara cells apoptosis and necrosis upon beta-1 integrin blocking in wt and p21 ko mice. A significant increase ($p < 0.05$) in apoptosis was observed upon beta-1 integrin blocking in Clara cells from wt and p21 ko mice at 72 and 120 hours in culture (A). No significant changes in necrosis were observed upon beta-1 block in cells from both wt and p21 ko mice (B). (D) represents the increase in apoptosis upon beta-1 blocked in cells from wt mice as compared to control (not beta-1 blocked) (C). Magnification x200.

6.5 Effect of beta 1 integrin blocking on cyclin kinase inhibitors p21, p27 and p53 in Clara cells in wt and p21 ko mice.

Three cyclin kinase inhibitors p21, p27 and p53 were studied by immunocytochemistry (Figures 6.6, 6.7, 6.8 & 6.9).

The expression of cytoplasmic p21 increased ($p < 0.05$) upon beta-1 integrin blocking at 120 hours in culture. The expression of nuclear p21 increased ($p < 0.05$) upon beta-1 integrin blocking at 72 and 120 hours in culture. The expression of nuclear p21 increased ($p < 0.05$) in culture from time 24 to 120 hours upon beta-1 integrin blocking.

A decrease ($p < 0.05$) in the expression of cytoplasmic p27 was observed in cells from wt mice at 24 hours in culture and in cells from p21 ko mice in all time points in culture, upon beta-1 integrin blocking. A significant increase ($p < 0.05$) in the nuclear p27 expression was observed in cells from p21 ko mice at 24 hours and in cells from wt mice at 72 hours in culture upon beta-1integrin blocking.

The expression of nuclear p53 increased significantly ($p < 0.05$) in cells from wt and p21 ko mice upon beta-1 integrin blocking at 72 and 120 hours in culture. Upon beta-1 blocking, there was an increase ($p < 0.05$) in the expression of nuclear p53 from 24 to 120 hours in culture in cells from both wt and p21 ko mice. No significant differences in the nuclear and cytoplasmic expression of p53 were noted in Clara cells from wt mice when compared to p21 ko mice. No significant changes in the expression of cytoplasmic p53 were observed upon beta-1 integrin blocking in cells from both wt and p21 ko mice.

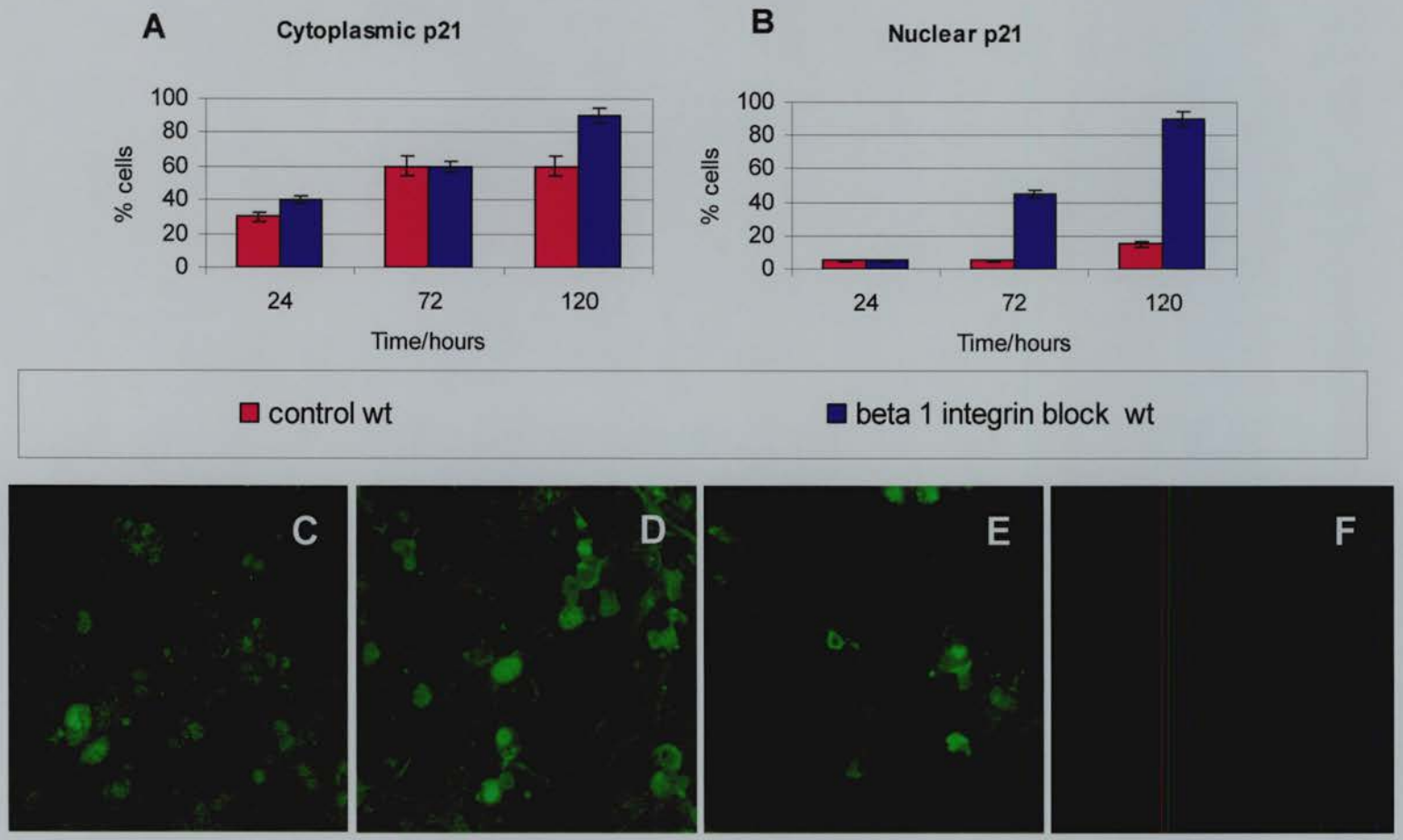

Figure 6.6 - Changes in the expression of p21 upon beta-1 integrin blocking in Clara cells from wt and p21 ko mice. The expression of cytoplasmic p21 increased ($p < 0.05$) upon beta-1 integrin blocking at 120 hours in culture [graph (A), control (not beta-1 integrin blocked) at 120 hours (C), beta-1 blocked cells at 120 hours (D)]. The expression of nuclear p21 increased ($p < 0.05$) upon beta-1 integrin blocking at 72 and 120 hours in culture (B) [graph (B), control (not beta-1 integrin blocked) at 120 hours (C), beta-1 integrin blocked cells at 120 hours (D)]. The expression of nuclear p21 increased ($p < 0.05$) in culture from time 24 to 120 hours upon beta-1 integrin blocking [graphs (A & B), beta-1 blocked cells at 24 hours (E), beta-1 blocked cells at 120 hours (D)]. F is a typical negative control for immunofluorescence whereby the primary antibody was omitted. Magnification x 200.

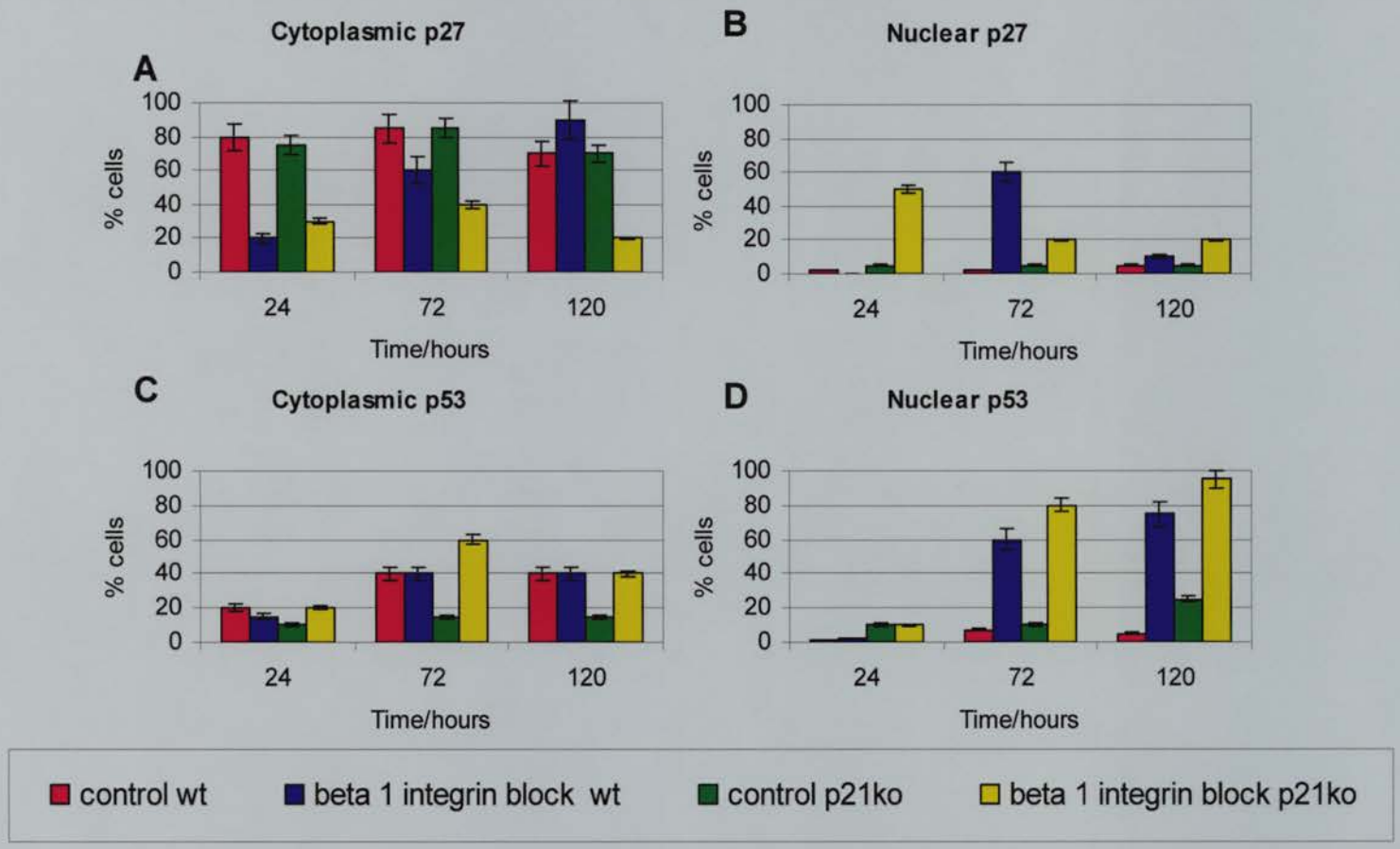

Figure 6.7 - Changes p27 and p53 expression upon beta-1 integrin blocking in Clara cells from wt and p21 ko mice. Upon beta-1 integrin blocking, there was a decrease ($p < 0.05$) in the expression of cytoplasmic p27 was observed in cells from wt mice at 24 hours in culture and in cells from p21 ko mice in all time point in culture (A). A significant increase ($p < 0.05$) in the nuclear p27 expression was observed in cells from p21 ko mice at 24 hours and in cells from wt mice at 72 hours in culture upon beta-1 blocking (B). No significant changes in the expression of cytoplasmic p53 were observed upon beta-1 blocking in cells from both wt and p21 ko mice (C). The expression of nuclear p53 increased significantly ($p < 0.05$) in cells from wt and p21 ko mice upon beta-1 integrin blocking at 72 and 120 hours in culture. An increase ($p < 0.05$) in the expression of nuclear p53 was observed from 24 to 120 hours in culture in cells from both wt and p21 ko mice (D).

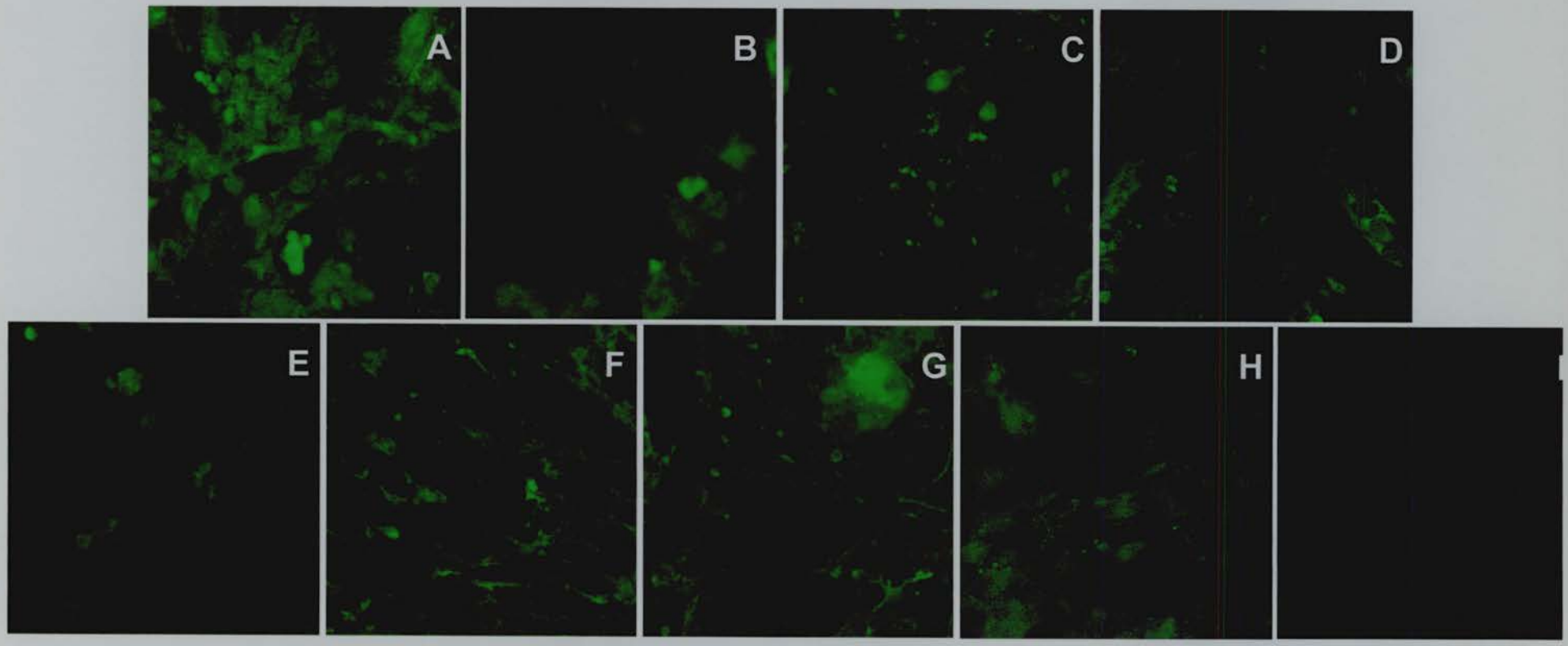

Figure 6.8 - Changes in p27 expression upon beta-1 integrin blocking in Clara cells from wt and p21 ko mice. Upon beta-1 integrin blocking, there was a decrease ($p < 0.05$) in the expression of cytoplasmic p27 in cells from wt mice at 24 hours in culture (A & B) and in cells from p21 ko mice at all time points in culture (cells from at 120 hours in culture are represented in C & D). A significant increase ($p < 0.05$) in the nuclear p27 expression was observed in cells from p21 ko mice at 24 hours (E & F) and in cells from wt mice at 72 hours (G & H) in culture upon beta-1 blocking. [A, C, E & G are cells from control (not beta-1 blocked, while B, D, F & H are beta-1 blocked cells]. I represent the negative control for immunofluorescence whereby the primary antibody was omitted. Magnification x 200.

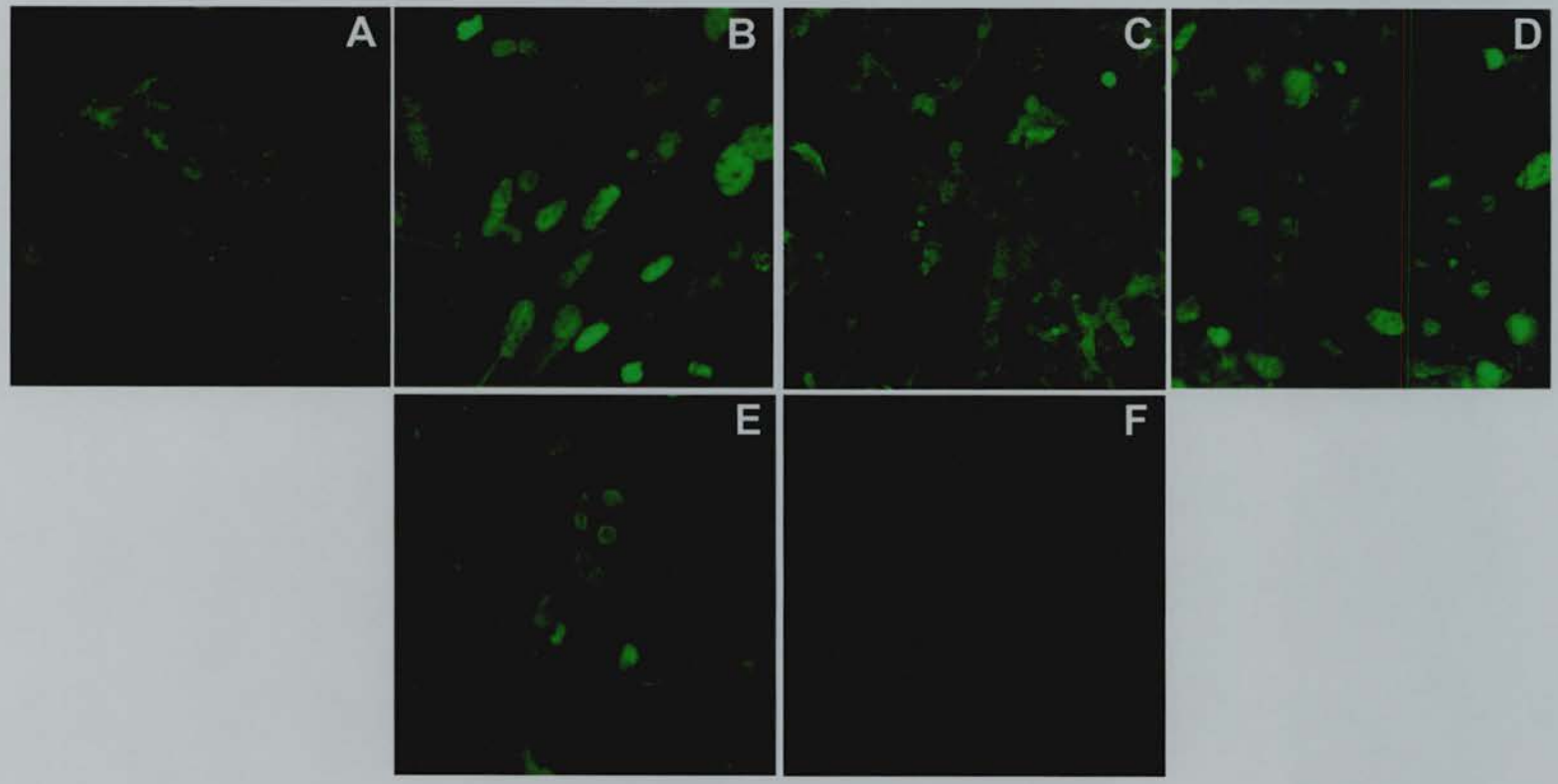

Figure 6.9 - Changes in p53 expression upon beta-1 integrin blocking in Clara cells from wt and p21 ko mice. Upon beta-1 integrin blocking, the expression of nuclear p53 increased significantly ($p < 0.05$) in cells from wt mice (A & B) and in cells from p21 ko mice (C & D) at 120 hours in culture. An increase ($p < 0.05$) in the expression of nuclear p53 was observed from 24 (E) to 120 (D) hours in culture in cells from p21 ko mice. No significant changes in the expression of cytoplasmic p53 were observed upon beta-1 integrin blocking in cells from both wt and p21 ko mice (A - E). [A & C are cells from control (not beta-1 integrin blocked, while B, D & E are beta-1 blocked cells]. F is the negative control for immunofluorescence whereby the primary antibody was omitted. Magnification x 200.

6.6 Discussion.

6.6.1 p21 is not involved in the expression of cytokeratins 8, 18 and 19 when Clara cells are attached to laminin via $\alpha_6\beta_4$.

No significant changes in the expression of cytokeratins 8, 18 and 19 were observed upon beta-1 blocking in Clara cells from both wt and p21 ko mice. Thus when Clara cells are attached to laminin via $\alpha_6\beta_4$, the presence or absence of p21 did not influence expression of cytokeratin 8, 18 and 19.

As seen in the results from the previous chapter (chapter 5), cytokeratin 8, 18 and 19 were lower at time 0 (unattached cells) in Clara cells from p21 ko mice. Thus it can be concluded that Clara cell interactions via $\alpha_6\beta_4$ are important for the expression of cytokeratin 8, 18 and 19 and p21 is most probably involved. Since as number of differences in the cytokeratins expression has been shown in lung cells and cytokeratins have been used a differentiation markers in lung epithelial cells (Gunning et al., 1992; Moll et al., 1982; Blobel et al., 1984; Moll, 1987) , it can hypothesised that Clara cell differentiation is influenced by cell-matrix adhesion through $\alpha_6\beta_4$ integrins.

The activation of p53 and p21 by β_4 integrin was previously described (Clarke et al., 1995; Bachelder et al., 1999a) and p53 was also found to inhibit survival signalling through $\alpha_6\beta_4$ integrin (Bachelder et al., 1999b). Thus most probably the levels of expression of both p21 and p53 are important in the control of cell differentiation. p21 has previously been shown to be able to both promote (Matsumura et al., 1997; Baccini et al., 2001; Liu et al., 1996a; Liu et al., 1996b; Hiraoka et al., 2002; Kramer et al., 2002; Park and Chung, 2001; Park and Chung, 2001; Billon et al., 1996) (Halevy et al., 1995; Nadal et al., 1997) and inhibit differentiation (Di Cunto et al.,

1998; Yamamoto et al., 1998). In the p21 null mice, normal differentiation has been observed thus implying that p21 is not a mutually exclusive agent that promotes differentiation (Paramio et al., 2001; Cox, 1997; McDonald et al., 1996; Deng et al., 1995).

Disruption of cell-$\alpha_6\beta_4$ interaction in Clara cells lacking p21 could lead to differentiation and maybe the generation of another cell type or an intermediate cell type. This could be one of the possible mechanisms by which Clara cells differentiate into other cells such as ciliated cells and it could also be a possible mechanism by which Clara cells respond after epithelial cells injury when neighbouring cells are damaged and maybe shed.

Cytokeratin 8 expression was lower ($p < 0.05$) in control Clara cells at 72 and 120 hours in cultures, from p21 ko mice. Cytokeratin 19 expression was also found to be lower ($p < 0.05$) in control Clara cells at 120 hours in cultures, from p21 ko mice. This decrease could be due beta-1 integrin interactions. Thus cell-beta-1 integrin interactions both in the absence and presence of p21, could influence cell differentiation.

Cytokeratin 18 expression was lower ($p < 0.05$) in beta-1 blocked Clara cells from wt mice at 72 hours in culture. As observed in chapter 5, the presence or absence of laminin in the ECM did not vary significantly the expression of cytokeratin 18. This could due to other factors influencing the expression of cytokeratin 18 such as cell-beta-1 integrin interactions.

6.6.2 Beta-1 integrin is involved in Clara cell proliferation.

Upon beta-1 integrin blocking, a decrease ($p < 0.05$) in BrdU incorporation was observed in cells from wt mice at 24 and 72 hours in culture, and in cells from p21 ko mice at 24 hours in culture. Thus the cell-beta-1 integrin interaction could be promoting proliferation in Clara cells and disruption of this interaction could result in a decrease in proliferation. β_1 integrin complexes has been previously shown to be involved in cell proliferation (Rosales and Juliano, 1995; Varner et al., 1995; Howe et al., 1998; Miyata et al., 2000; Carloni et al., 1998; Zhang et al., 1995). $\alpha_5\beta_1$, a fibronectin binding integrin, was found to decrease cell proliferation in the absence of attachment to fibronectin and that ligation of this receptor with fibronectin reverses this process (Varner et al., 1995). Trypsin was shown to stimulate the integrin $\alpha_5\beta_1$ dependent adhesion to human gastric carcinoma cells to fibronectin and increases cell proliferation (Miyata et al., 2000). Since trypsin was used to isolate Clara cells, the $\alpha_5\beta_1$ integrin complexes could have been stimulated but since beta-1 integrin was blocked and thus was not bound to a suitable receptor, there was a decrease in cell proliferation.

6.6.3 Apoptosis rate increases upon beta-1 integrin blocking.

Upon beta-1 integrin blocking, an increase in apoptosis was observed in Clara cells from both wt and p21 ko mice at 72 and 120 hours in culture. This result was expected since a number of studies have shown that when cells are detached from substrate or anchorage is presented they usually undergo apoptosis (Ruoslahti and Reed, 1994; Frisch and Ruoslahti, 1997; Frisch and Francis, 1994; Schwartz, 1997; Assoian, 1997; Day et al., 1997; Meredith et al., 1993; McGill et al., 1997; Ilic et al., 1998; Vitale et al., 1999; Kettritz et al., 1999; Sethi et al., 1999; Montgomery et al.,

1994; Clarke et al., 1995). Thus disruption of Clara cell- beta-1 integrin interactions results in apoptosis.

Upon beta-1 integrin blocking, the apoptosis rate was higher ($p < 0.05$) in cells from wt mice compared to cells from p21 ko mice at 72 hours. In the absence of p21, there was a reduction of the apoptosis rate, thus p21 seems to induce apoptosis in Clara cell culture upon beta-1 integrin blocking. p21 has been previously shown to be able to both induce (Kondo et al., 1997; Fotedar et al., 1999) and inhibit apoptosis (Polyak et al., 1996; Gorospe et al., 1998; Wang et al., 2000; Gorospe et al., 1997; Mahyar-Roemer and Roemer, 2001).

6.6.4 PCNA expression is increased upon beta-1 integrin blocking but PCNA requires p21 for its nuclear localisation.

Interesting results were obtained with regards to the PCNA expression. Cytoplasmic PCNA expression was found to be higher ($p < 0.05$) in cells from both wt and p21 ko mice upon beta-1 integrin blocking at 120 hours. The expression of nuclear PCNA expression significantly increased ($p < 0.05$) in cells from Clara cells from wt mice only upon beta-1 blocking at 72 and 120 hours in culture. In the absence of p21, there was an increase in cytoplasmic PCNA expression but not nuclear PCNA expression. Thus upon beta-1 blocking the expression of PCNA seems to be increase in cells from both wt and p21 ko mice but p21 is essential in the nuclear PCNA localisation. The role of p21 in the nuclear localisation of PCNA and the importance of this complex in cell cycle progression have been previously described (Li et al., 1994; Li et al., 1995a; Vairapandi et al., 1996; Funk et al., 1997; Warbrick et al., 1997; Chuang et al., 1997; Cayrol et al., 1998; Oku et al., 1998; Rousseau et al., 1999).

6.6.5 Nuclear p21 and p53 expression increase upon beta-1 integrin blocking in Clara cells culture.

Upon beta-1 integrin blocking, an increase ($p < 0.05$) in the nuclear p21 expression was observed in Clara cells. p21 expression was previously shown to be increased by the beta-4 cytoplasmic domain. Lower levels of $\alpha_6\beta_4$ surface expression were shown to induce p21 sufficient to induce partial G1 arrest and some apoptosis, while higher levels of $\alpha_6\beta_4$ expression resulted in more p21 expression and more widespread apoptotic death (Clarke et al., 1995). Thus in this case, due to the fact that beta-1 integrin was blocked, there could have been a hypothetical increase in either the expression of $\alpha_6\beta_4$ or relatively more $\alpha_6\beta_4$ integrin adhered to an appropriate receptor and thus results in an increase in p21. More work needs to be carried out, to find out the exact mechanism by which p21 expression increases upon beta-1 integrin blocking. The literature about this topic is so far very limited.

Upon beta-1 integrin blocking, the nuclear p53 expression increased ($p < 0.05$) in Clara cells from both wt and p21 ko mice. $\alpha_6\beta_4$ was shown to activate p53 (Bachelder et al., 1999a), whereas p53 itself was found to inhibit $\alpha_6\beta_4$ integrin survival signalling (Bachelder et al., 1999b). The reason for the increase in the nuclear p53 could be the same as for the p21, but whether the increase of p21 is dependent or independent of p53 is not known.

In this study no significant differences in the nuclear and cytoplasmic expression of p53 were observed in Clara cells in the presence or absence of p21 upon beta-1 blocking. Results from the previous section (Chapter 5) suggest, that in the absence of p21 there was an increase in p53. It seems that apart from beta-1 and beta-4 integrins, other integrins are involved, possibly beta-3, beta-6 or beta-7 (see Table 1.2, Introduction). Although beta-3 was found not to be present in lung epithelium cells (results from Chapter 4) and others (Damjanovich et al., 1992; Pilewski et al., 1997) and in Clara cell cultures (Chapter 4), beta-3 expression was found to be present in the epithelial cells of fibrotic lungs (Huang et al., 1996; Munger et al.,

1999; Kaminski et al., 2000; Sheppard, 2000; Sheppard, 2001a; Sheppard, 2001b; Reynolds et al., 2002). Beta-3 integrin was shown to be involved in the p53 and p21 expressions (Montgomery et al., 1994; Strömblad et al., 1996). α_v complexes either to β_3, β_5 or β_8 were also found to be involved in the expression of p53 and p21 (Strömblad et al., 2002; Milner et al., 1999; Cambier et al., 2000; Arroyo et al., 2000; Häkkinen et al., 2000). Thus, it can be hypothesised that the increase in p53 expression in the absence of p21 could be mediated via integrins, but further studies have to be carried out to determine which integrins are involved and to determine the exact mechanism.

6.6.6 p27 translocation to the nucleus occurs faster in the absence of p21 upon beta-1 integrin in Clara cells.

Upon beta-1 integrin blocking, there was a decrease ($p < 0.05$) in the expression of cytoplasmic p27 at all time points in culture. An increase in nuclear p27 expression in cells from p21 ko mice at 24 hours and in cells from wt mice at 72 hours in culture, were observed upon beta-1 integrin blocking. Thus upon beta-1 integrin blocking, the translocation of p27 from the cytoplasm to the nucleus seems to be faster in the absence of p21. It has previously been hypothesised that p27 expression could be influenced via beta-1 integrin (Fornaro et al., 1999; Bao et al., 2002; Henriet et al., 2000), but the exact mechanism is still unclear, and the literature so far is quite limited. Thus further studies need to be carried out to describe the relationship of p27 with integrins.

6.6.7 Conclusion

When cells are attached to $\alpha_6\beta_4$, p21 is not involved in the cytokeratins 8, 18 and 19 expressions of Clara cells, while when cell-$\alpha_6\beta_4$ interaction is disturbed the presence of p21 is important for the cytokeratins expression. Cell-beta-1 interactions could also be involved in the cytokeratins expressions. Therefore the expression levels of p21 could be a determinant factor in Clara cell differentiation. Cell-beta 1 interactions were described as a potential factor in cell proliferation. The lack of binding of beta-1 integrin to an appropriate receptor could lead to a decrease in cell proliferation.

Beta-1 integrin disruption could also lead to an increase in PCNA expression but for an increase in nuclear PCNA p21 was shown to be an essential factor. Upon blocking beta-1 integrin there is an increased nuclear expression of p21, p27 and p53 and an increase in apoptosis rate.

Thus as originally hypothesised, integrins that bind to laminin are important for cell cycle progression through p21-dependent or -independent mechanisms.

Chapter 7 - Cytoplasmic and nuclear $p21^{Waf1/Cip1}$.

7.1 Introduction

The functions of $p21^{Waf1/Cip1}$ are complex and contradictory. $p21^{Waf1/Cip1}$ was shown to promote apoptosis (McKay et al., 1998; Gervais et al., 2000), protect cells from undergoing apoptosis (McKay et al., 1998; Lu et al., 1998; Bulavin et al., 1999; Asada et al., 1999; Zhang et al., 1999; Gervais et al., 2000), to promote proliferation (Mantel et al., 1996; Lai et al., 2002), inhibit proliferation (Balomenos et al., 2000; Sugibayashi et al., 2002; Harper et al., 1993; El-Deiry et al., 1993; Harper et al., 1995), inhibit differentiation (Yamamoto et al., 1998; Harvat et al., 1998) and promote differentiation (Steinman et al., 1994; Liu et al., 1996a; Liu et al., 1996b; Billon et al., 1996; Matsumura et al., 1997; Nadal et al., 1997).

The cell-cycle inhibitory activity of $p21^{Cip1/Waf1}$ is associated with its nuclear localisation. However a number of cytoplasmic forms of p21 have been described. So far six different forms of cytoplasmic p21 have been described each of which have different sizes: 21 kDa protein (Asada et al., 1999), 20 kDa protein (Tchou et al., 1996), 19 kDa protein (Poon and Hunter, 1998), 16 kDa protein (Donato and Perez, 1998), 15 kDa protein (Zhang et al., 1999) and a 14 kDa protein (Jin et al., 2000; Gervais et al., 2000).

A physical association between cytoplasmic p21 and ASK1 was described and was found to suppress the activity of ASK 1 and MAPK (SAPK/JNK) cascade activation thus preventing the cell from undergoing apoptosis (Asada et al., 1999). It has been suggested that cytoplasmic p21 is formed by cleavage or truncation during apoptosis. (Prabhu et al., 1997; Donato and Perez, 1998; Gervais et al., 2000; Jin et al., 2000; Zhang et al., 1999). The role of cytoplasmic p21 is still unclear (Dotto, 2000).

Changes in expression of p21 were shown to be involved in animal models of lung diseases such as asthma and fibrosis (chapter 3) and its regulation and expression could be affected by disruption of cell-matrix interactions (as shown in chapter 5 and 6). Thus by studying cytoplasmic and nuclear p21 and their relative complexes, one might understand the actual pathways by which p21 might be involved in the injury and repair processes of the lung. The main objectives of this chapter are to describe the cytoplasmic and nuclear p21 proteins found in Clara cell cultures and to try to describe their functional roles in terms of the complexes formation.

7.2 Cytoplasmic and nuclear p21 proteins in Clara cells.

As shown previously (results chapters 3, 4, 5 and 6), p21 was observed in both the cytoplasm and nucleus. An important question to ask is to determine whether the protein seen in the cytoplasm was the same as the one found in the nucleus. Cytoplasmic and nuclear protein extractions were carried out from cultured Clara cells at 72 hours. The extracts of which were separated on an appropriate gel and blotted using a p21 antibody raised against the full-length p21. Protein extracts from Clara cells from p21ko mice were used as the negative control.

The nuclear extract had a 20kDa protein that corresponds to the full-length p21, while the cytoplasmic extract had a 17 kDa protein (Figure 7.1 A).

7.3 Cytoplasmic p21 is a 17kDa protein and is truncated at the C-terminus.

The next aim was to determine whether the cytoplasmic form is truncated and where the truncation is, that is either towards the N-terminal or towards the C-terminal. Total protein extracts from cultured Clara cells at 72 hours were immunoprecipitated

with either an antibody raised against the full-length p21 or towards the C-terminus of p21. Protein extracts from Clara cells from p21ko mice were used as the negative control.

Two proteins were obtained when immunoprecipitated with the antibody raised against full-length p21, the 20 and 17kDa proteins that were previously observed. Only the 20 kDa was obtained when immunoprecipitated with the antibody raised against the C-terminus of p21. Thus the cytoplasmic p21 is a 17 kDa protein and is truncated in the C-terminus (Figure 7.1 B).

7.4 Complexes of cytoplasmic and nuclear $p21^{Waf1/Cip1}$.

The next question was to determine the cytoplasmic and nuclear p21 complexes. Total protein extracts from cultured Clara cells at 72 hours were immunoprecipitated with a number of antibodies, namely PCNA, cdk2, cdk4, cdk6, cyclin D3 and cyclin E. The immunoprecipitated proteins were then blotted using antibody raised against the full-length p21 protein. Protein extracts from Clara cells from p21ko mice were used as the negative control.

Nuclear p21 protein (20 kDa) was found to form complexes with PCNA, cdk 2, cdk 4, cdk 6, cyclin D3 and cyclin E. Cytoplasmic p21 protein (17 kDa) was found to form complexes with cdk 4 and cyclin D3 (Figure 7.1 C).

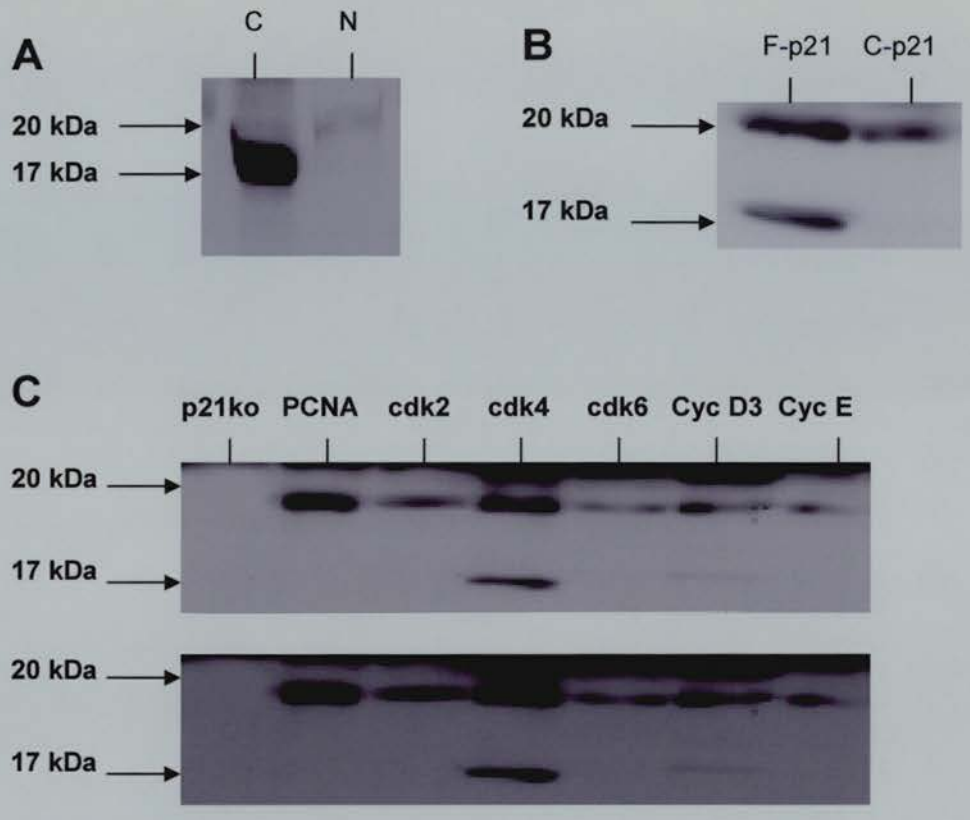

Figure 7.1 - Cytoplasmic and Nuclear p21. Cytoplasmic and nuclear protein extracts from Clara cells at 72 hours in culture were blotted against an antibody raised against full length p21 (F-p21). Cytoplasmic p21 was found to be a 17 kDa protein while nuclear p21 was found to be 20 kDa protein (A). Total protein extracts from Clara cells at 72 hours culture were blotted against either F-p21 or an antibody raised against the C-terminal of p21 (C-p21). The two proteins were observed when F-p21 was used, but only the 20 kDa protein was observed when C-p21 was used, thus the 17 kDa protein (cytoplasmic) is C-terminal truncated (B). Total protein extracts from Clara cells at 72 hours culture were immunoprecipitated against a number of antibodies and blotted against F-p21. Nuclear p21 (20 kDa) was found complexed with PCNA, cdk2, cdk4, cdk6, cyclin D3 and cyclin E, while cytoplasmic p21 (17 kDa) was found complexed with cdk 4 and cyclin D3 (C). [Top membrane in C was exposed for 10 minutes, while lower membrane in C was exposed for 3 hours.]

7.5 Discussion.

Two types of p21 were found in cultured Clara cells, a 20 kDa and 17 kDa protein. The 20 kDa is the full-length p21 protein (20 kDa in mouse while 21 kDa in human) while the 17 kDa is a C-terminal truncated form of p21. The 20 kDa is found in the nucleus while the 17 kDa protein is found in the cytoplasm. The nuclear localisation signal (NLS) of p21 is localised at the C-terminal of p21 (Dotto, 2000), thus the truncated 17 kDa cytoplasmic p21 does not have a NLS and maybe this is the reason for its localisation.

The 17 kDa protein obtained from Clara cell cultures seems to be a different protein from the cytoplasmic p21 described in the literature so far. Six different forms of cytoplasmic p21 have been described, having different sizes varying from 21 kDa protein (Asada et al., 1999), 20 kDa protein (Tchou et al., 1996), 19 kDa protein (Poon and Hunter, 1998), 16 kDa protein (Donato and Perez, 1998), 15 kDa protein (Zhang et al., 1999) to a 14 kDa protein (Jin et al., 2000; Gervais et al., 2000).

The cytoplasmic 21 kDa protein described by Asada et al., (1999), is most probably located in the cytoplasm due to a mutation in the nuclear localisation signal, was found to complex with MEKKs (ASK1) and thus inhibited apoptosis. The 15 kDa (Zhang et al., 1999) and the 14 kDa (Gervais et al., 2000; Zhang et al., 1999) p21 proteins are in the cytoplasm as a consequence of caspase-dependent cleavage of its nuclear C-terminus domain and are unable to suppress growth as well as apoptosis. This truncation would also compromise the ability of p21 to promote cyclin/CDK nuclear localisation, with the same biological end point effect (Gervais et al., 2000). The 16 kDa cytoplasmic p21 protein described by Donato and Perez, (1998) resulted from TNF-induced proteolysis by an apoptotic protease. The 14 kDa cytoplasmic p21 described by Jin et al., (2000) was found to occur due to proteolytic cleavage of p21 that is associated with cyclin A-cdk2 which is a major mechanism for up-regulation a cyclin A-Cdk2 kinases activity in the early stages of apoptosis. 20 kDa (Tchou et al., 1996) and 19 kDa (Poon and Hunter, 1998; Orend et al., 1998) proteins

truncated at the C-terminal and localised in the cytoplasm due to insufficient signals for nuclear localisation were described. The 19 kDa p21 protein described by (Poon and Hunter, 1998) was found bound to cyclin E-cdk 2 (Orend et al., 1998).

Mutated p21 lacking Cdk-inhibitory activity was found not to be able to prevent apoptosis in human colorectal carcinoma cells (Lu et al., 1998). The expression of either the N- and C-terminal regions of p21 was found to inhibit DNA synthesis and cell growth, but not as efficiently as full length p21. The effectiveness of the two domains was found to be dependable on the stability via the ubiquitin-proteasome pathway (Rousseau et al., 1999).

The mouse homologue of p21 has been identified as a 20 kDa protein (Gu et al., 1993). As expected, the 20 kDa (full length) p21 was found to form complexes with PCNA, cdk 2, cdk 4, cdk 6, cyclin D3 or cyclin E. The truncation of the 17 kDa cytoplasmic protein was found to bind with cyclin D3 and cdk 4. Such a complex has not been described before.

Over-expression of positive regulators including cyclin D3 and cdk4 is thought to contribute to cancer of the breast, colon and other tissues (Sherr, 1996; Suhardja et al., 1999; Ortega et al., 2002; Wong et al., 2001). Thus inhibitors of cdks are anticipated to posess therapeutic utility against a wide variety of proliferative diseases, especially cancer (Toogood, 2001).

Cdk4/6-cyclin D complexes help cells to pass through the restriction point (R) in the cell cycle and drive them from early to late G1 phase (Grana and Reddy, 1995; Nigg, 1995; Paggi et al., 1996; Sherr, 1996; Elledge, 1996; Beijersbergen and Bernards, 1996; Nurse et al., 1998; Sherr, 1995). This is accomplished by two independent mechanisms. First, Cdk4/6-cyclin D phosphorylates pRb, which results in activation of E2F transcription factors required for the synthesis of cyclin E (Beijersbergen and Bernards, 1996; Paggi et al., 1996; Herwig and Strauss, 1997; Philips et al., 1997; Dosaka-Akita et al., 1997; Day et al., 1997; Amellem et al., 1998; Marchetti et al., 1998). Secondly, Cdk4/6-cyclin D complexes bind to the Cip/Kip family of cell

cycle inhibitors, whose primary role is to inhibit Cdk2-cyclin D-Cip/Kip complexes which contributes to G1 progression by the removal of these inhibitors and thus preventing them from binding and inactivating Cdk2-cyclin E complexes (Cheng et al., 1999; Grana and Reddy, 1995; Hiyama et al., 1997; Ekholm and Reed, 2000; Grana and Reddy, 1995; Hiyama et al., 1997; Ortega et al., 2002).

p21 promotes the assembly of Cdk4/6 and cyclin D *in vitro* and associates with cyclinD/Cdk4 complexes during cell-cycle progression (LaBaer et al., 1997; Morisaki et al., 1999; Cheng et al., 1999; Wong et al., 2001). p21 was also shown to complex with cdk2 and thus lead to growth arrest (Niculescu et al., 1998; Shiyanov et al., 1996; Jones et al., 1997; Orend et al., 1998; Morisaki et al., 1999; Aikawa et al., 2001). The role of p21 as an assembly activator or inhibitor depends on its expression level. At low and intermediate concentrations it is an assembly factor, while at high concentrations it is an inhibitor (LaBaer et al., 1997; Hiyama et al., 1997; Morisaki et al., 1999).

Primary mouse embryonic fibroblasts cells from p21 ko and p27 ko mice, failed to assemble detectable amounts of cyclin D-Cdk complexes, express cyclin D proteins at a reduced levels, and are unable to efficiently direct cyclin D proteins to the cell nucleus (Cheng et al., 1999). On the other hand, it was suggested that neither p21 nor p27 are required for the formation of cyclin D3-cdk4 complexes and that cyclin D3-cdk4 complexes containing p21 or p27 are inactive. Thus it is unlikely that the formation of these complexes would be dependent on their association with proteins that would ultimately prevent this activation (Bagui et al., 2000). Thus, it is still unclear to what extent sequestering of p21 inhibitor by Cdk4/cyclin D complexes contributes to G1 progression (Ortega et al., 2002).

A complex of cdk4/cyclin D with cytoplasmic p21 was never previously described. There are a number of possibilities how p21 can regulate the cell cycle through the cdk 4/cyclin D complexes. There is no direct proof that the cytoplasmic p21 protein is derived directly from p21 but RT-PCR/Southern analysis of p21 mRNA argues against the possibility that the cytoplasmic p21 protein is encoded from an

alternatively spliced mRNA (Tchou et al., 1996). The truncation of the p21 could occur at either the nucleus or the cytoplasm. If the truncation occurs within the nucleus the truncated p21 could possibly bind to the cdk 4/cyclin D3 complex or to cyclin D3 or cdk4 individually and exporting them out of the nucleus to the cytoplasm. p21 truncation could also occur in the cytoplasm. Since both cdk 4 and cyclin D3 lack a nuclear localisation signal, if either cdk 4 or cyclin D3 or cdk4/cyclin D3 complexes bind to the cytoplasmic p21 protein, they cannot be transported to the nucleus thus most probably they would be inactive and would localised in the cytoplasm.

Based on these data, it can be hypothesised that the functional role of the 17 kDa cytoplasmic is to inhibit DNA synthesis and cell growth via the inactivation of cdk 4/cyclin D3 complexes and therefore suppression of the E2F activity by preventing the phosphorylation of the retinoblastoma protein. Since cdk 4/cyclin D3 complexes are also involved in promoting apoptosis pathways, cytoplasmic p21 could also be involved in the control apoptosis processes.

Chapter 8 - Conclusions.

Changing p21 expression was seen in three animal models of lung disease. Due to limited availability of time points and tissue the significance of these observations is unknown. All that can be said is that p21 may have a role in lung diseases and in the regulation of epithelial cell proliferation, differentiation and death.

However, p21 status was found to effect Clara cell differentiation modulation through laminin receptors. This may be an indirect action. External stimuli such as cell-matrix interactions had an effect on expression of p21 in Clara cells *in vitro.* The presence of p21 was also shown to be important for the increase in apoptosis of Clara cells. In the absence of p21, the expression levels of p53 were higher implying a compensatory mechanism for the lack of p21. The up-regulation of p21 through p53 dependent pathway is well described in the literature but the actual mechanism for the up-regulation of p53 in the absence of p21 is unclear.

p21 status affected Clara cell proliferation and apoptosis triggered through beta-1 integrin blocking. p21 was shown to be important for PCNA nuclear localisation and cell-beta 1 integrin disruption leads to an increase in PCNA expression. Disruption of cell-matrix interaction by blocking beta 1 integrin was shown to increase apoptosis and decrease proliferation in Clara cell cultures. p21 and p53 were upregulated upon beta 1 integrin blocking. Thus as originally hypothesised, Clara cells respond to changes in the environment through integrin mediated changes in p21. Upon beta 1 integrin blocking and in the absence of p21, the nuclear localisation of p27 occurs faster. This could be another compensatory mechanism for the lack of p21 in Clara cells *in vitro* upon disruption of cell-matrix interaction.

Two forms of p21 were found in Clara cells; a nuclear and a cytoplasmic form. The cytoplasmic p21 was found to be a 17 kDa truncated protein of the full-length p21. The cytoplasmic p21 protein seemed to be a novel form of p21 compared to cytoplasmic p21 proteins previously described in the literature. From pull-down

assays it was determined that nuclear and cytoplasmic p21 bind to different proteins. The cytoplasmic p21 is most probably functionally active and inhibits the cell cycle progression at the G1 phase of the cell cycle and via the inactivation of cdk4/cyclin D complexes and therefore suppression of the E2F activity.

Future Work.

Although a number of important questions have been answered, there are still an even larger number of questions which need to be investigated. In this thesis, significant changes in Clara cell cultures were obtained by altering the cell-matrix interactions, for example by beta 1 integrin signal blocking. Thus it would be interesting use beta 1 integrin blocking in an *in vivo* animal model of lung disease such as fibrosis or asthma to see whether this treatment modulates cell growth and affects disease outcome.

Since the 17 kDa cytoplasmic p21 is probably a novel protein and was shown to have a functional role, it would be important to investigate it further. This might be done by generating a p21 knockout mouse expressing the 17 kDa protein as a transgene.

Another interesting aspect which needs further studies is the importance of cell-cell interactions. This includes both epithelial cells interacting with each other as well as the interactions of epithelial cells with other cells including inflammatory cells.

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APPENDICES

APPENDIX 1 - Buffers and solutions.

Bouin's fixative

425ml methanol

25ml glacial acetic acid

50 ml 40% formalin

Cytoplasmic extraction buffer

50mM sodium fluoride

5mM tetra sodium pyrophosphate

1mM sodium orthovanadate

10mM β-glyceropyrophosphate

0.5% NP-40

2mM EDTA

20mM disodium hydrogen phosphate

20mM monosodium dihydrogen phosphate

Protease inhibitor (1 cocktail tablet per 10ml) Boehringer Mannheim

Nuclear extraction buffer

50mM sodium fluoride

5mM tetra sodium pyrophosphate

1mM sodium orthovanadate

10mM β-glyceropyrophosphate

0.5% NP-40

2mM EDTA

20mM disodium hydrogen phosphate

20mM monosodium dihydrogen phosphate

300mM sodium chloride

Protease inhibitor (1 cocktail tablet per 10ml) Boehringer Mannheim

PBS

9g NaCl

0.262g monobasic sodium phosphate monohydrate (RMM 138)

1.15g dibasic sodium phosphate (RMM 142)

made up to 1 litre with ddH_2O, pH7.4, autoclaved

Tween PBS

PBS with 0.1% Tween-20

TBS

6.057g NaCl

8.709g Tris-HCl

made up to 1 litres with ddH_2O, pH 7.6 with HCl

TBST

TBS with 0.1% Tween-20

Towbin transfer buffer (pH 8.2-8.4)

25mM Tris (30.3 g, RMM 121.1)

192mM glycine (144.1g, RMM 75.07)

10g SDS

make up to 8 litres with ddH_2O add 2 litres of methanol

Do not add acid or base to adjust pH

Hydrogen peroxide blocking solution

3ml hydrogen peroxide (30% w/w stock, Sigma)

97ml ddH_2O

DAB substrate solution

made up fresh prior to use.

use 4.8ml of DAB buffer:

24ml of 0.2M Tris

38ml of 0.1M HCl

0.0681g imidazole

38ml ddH_2O

pH adjusted to pH 7.6

use 100µl DAB substrate

100µl frozen aliquots at 25mg/ml (50x stock)

add 100µl of 1% hydrogen peroxide:

10µl hydrogen peroxide (30% w/w stock, Sigma) in 290µl ddH_2O

Schiffs reagent

Make up in fume hood

2.5g Feulgen grade Fuchsine in 500ml boiled water

cool to 50°C and add 5g potassium metabisulphate

cool to room temp and add 10% HCl mix, 2g activated charcoal and leave overnight in dark

filter through Whatman filter paper and store at 4°C.

TAE 50x

242gTris base

57.1 ml glacial acetic acid

100 ml, EDTA (0.5M, pH 8.0)

made up to 1 litre with ddH_2O

Running Buffer

29g Tris Base

144g Glycine

10g SDS

made up to 1 litre with ddH_20

6 x sample treatment buffer (SDS-PAGE solution)

7ml 0.5M Tris-Cl, pH 6.8

1g SDS

3ml glycerol

0.93g DTT

1.2 mg Bromophenol Blue

dissolve and aliquot into 1ml and stored at $-70^{o}C$.

+Ca/Mg solution

500ml 0.9% sodium chloride

10ml 0.11M calcium chloride

20ml 0.15M potassium chloride

5ml 0.15M magnesium sulphate

15ml 0.1M phosphate buffer pH 7.4

30ml 0.2M HEPES

0.63g glucose

adjust pH to 7.4 and filter sterilize

-Ca/Mg solution

500ml 0.9% sodium chloride

20ml 0.15M potassium chloride

15ml 0.1M phosphate buffer pH 7.4

30ml 0.2M HEPES

0.63g glucose

adjust pH to 7.4 and filter sterilize

Trypsin

0.05g trypsin

20ml +Ca/Mg solution

filter sterilize

DNase 1 solution

0.01875g DNase I

75ml –Ca/Mg solution

filter sterilize

DNase 2 solution

0.0025g DNase I

50ml – Ca/Mg solution

filter sterilize

RIPA buffer

50 mM NaCl

1% NP-40

12 mM deoxycholate

3 mM SDS

50 mM Tris-HCl, pH 7.5

Protease inhibitor (1 cocktail tablet per 10ml) Boehringer Mannheim

Methocarn fixative

100 ml methanol

50 ml chloroform

25 ml glacial acetic acid

APPENDIX 2 - Suppliers.

Affymetrix,

Santa Clara, California, USA

Amersham Pharmacia Biotech

Amersham Place, Bucks, UK

Becton Dickinson UK

Oxford, UK

Bio-Rad Laboratories Ltd.

Bio-Rad House, Herts, UK

Boehringer Mannheim

Diagnostics and Biochemicals, East Sussex, UK

Chemicon

Chemicon International, Harrow, UK

CytoSignal

Affiniti Research Products Ltd., Dexon, UK

Dako

Bucke, UK

Gibco BRL

Life Technologies Limited, Paisley, UK

Hybaid

Thermo Life Sciences, Hampshire, UK

ICN Biomedicals Ltd.,

Buckinghamshire, UK

Microsoft Corporations

California, USA

Molecular Probes

Cambridge Bioscience, Cambridge, UK

Novex

CN Bioscience Ltd., Nottingham, UK

PharMingen

San Diego, California, USA

Promega

Southampton, UK

Santa Cruz Biotechnology

Santa Cruz, California, USA

Scientific Laboratory Supplies

Wilford, Nottingham, UK

Sigma UK

Sigma-Aldrich Company Ltd., Dorset, UK

Vector Labs Ltd

Peterborough, UK

PUBLICATIONS.

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Characterisation of lectin binding patterns of mouse bronchiolar and rat alveolar epithelial cells in culture

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Summary

Lung epithelial cell differentiation pathways remain unclear. This is due in part to the plasticity of these cells and the lack of markers which accurately reflect their differentiation status. The aim of this study was to determine if lectin binding properties are useful determinants of functional differentiation status *in vitro*. Mouse Clara cells were cultured for 5 days. During this time, no alteration in differentiation was evident by electron microscopy. No significant alteration in binding reactivity of *Bauhinia purpurea* (BPA), *Maclura pomifera* (MPA), Concanavalin A, Wheat germ or Helix pomatia lectins occurred in cultures compared with Clara cells in mouse lung tissue. In contrast, nitrotetrazolium blue reductase activity and CC10 expression declined in culture. Rat type II cells were cultured for 8 days. Between days 0 and 4, the number of type II cells identified by electron microscopy was constant at 70–80%, decreasing to 8% by day 6. In contrast, by day 4, only 42% cells retained alkaline phosphatase activity. BPA and MPA reactivity was altered at day 0 and day 4 respectively, compared with cells *in situ*. Therefore, the reactivity of lectins analysed here does not reflect functional differentiation status of cultured mouse Clara cells. However, BPA and MPA reactivity may be a sensitive indicator of alterations in rat type II cell differentiation *in vitro*.

Introduction

The bronchiolar and alveolar regions of the lung are lined by a single layer of epithelial cells. At least 8 subpopulations have been described within this epithelium, each with important, and often overlapping roles in lung function. For example, bronchiolar Clara cells and alveolar type II cells store and secrete components of the protective mucous and surfactant layers respectively. They contain high levels of biotransforming enzymes involved in detoxification, but also activation, of certain compounds (Myles *et al.* 1989, Kudo 1994, Quondamatteo *et al.* 1998). In addition, they both serve as progenitor cells in their respective regions (Massaro 1989, Voelker & Mason 1989). Methods for isolating relatively pure populations of Clara and type II cells have been described for several species, including human, providing excellent opportunities to study specific cell functions described above. However, the propensity of both of these cell types for rapid differentiation and loss of characteristic features once placed in culture has hindered full exploitation of these model systems. In addition, the identities of the cell types which subsequently arise in culture are, as yet, unclear. A lack of cell specific markers along with the ability of certain lung epithelial cell types apparently to differentiate, dedifferentiate and redifferentiate under various conditions (Johnson *et al.* 1990), has meant that cultures of such cells are poorly defined. Consequently, studies have mainly focused on *in vivo* animal experiments and more stable transformed cell lines.

Lectins have been used as tools to distinguish between lung cell types on the basis of their binding to specific carbohydrate groups, usually at the cell surface (Geleff *et al.* 1986, Tatrai *et al.* 1994). These lectin binding sites may be involved in intracellular recognition (Sato & Muramatsu 1985) and binding of yeast, bacteria and other microorganisms to host cells (Mouricout 1997). For example, *Maclura pomifera* agglutinin (MPA) binds to galactose (α-D-Gal) or *N*-acetylgalactosamine (α-D-GalNAc) residues while Concanavalin A (ConA) binds to glucose (α-D-Glc) and mannose (α-D-Man) residues. The specific nature of these interactions means that lectins are potentially useful markers for early epithelial changes in lung disease (Kasper *et al.* 1994) and ontological studies (Joyce-Brady & Brody 1990) as well as markers for *in vitro* differentiation investigations. However, as yet, few such *in vitro* studies have been carried out. Furthermore, many of the reports of lectin binding to either lung tissue or cultured cells published so far are contradictory.

For example, Brandt (1982) reported that ConA bound to type II cells in rat lung tissue while Dixon and Jersild (1983) and Williams (1984) found little to no binding of ConA to these cells. Similarly, Brandt (1982) found that *Ricinus communis* (RCA) bound to type I but not type II cells in rat lung tissue while Taatjes *et al.* (1990) observed RCA binding to both type I and type II cells in rat lung. In addition, in cultured rat type II cells, Dobbs *et al.* (1985) reported that MPA was bound progressively less and RCA bound more over time in culture while Kovacikova *et al.* (1999) observed no change in the levels of MPA or RCA binding to these cells in culture. Interspecies variations are also apparent with *Buahinia purpurea* agglutinin (BPA) reportedly binding to both type I and type II cells in human lung (Sarker *et al.* 1994) but only to type I cells in rat lung tissue (Kasper *et al.* 1994).

In an attempt to clarify this contentious area and to assess the potential of lectins as markers in primary lung cell culture studies, we have examined the binding of a panel of lectins to rat lung tissue as well as cultured rat type II cells in conjunction with measurements of alkaline phosphatase acitivity as an indicator of cell function, and ultrastructural characterisation by electron microscopy (EM). We have also carried out the first in-depth study of lectin binding to cultured mouse Clara cells, again in parallel with analyses of other Clara cell biochemical markers and EM to further characterise the phenotypes of mouse Clara cells and their derivatives in culture.

Materials and methods

Animals

Mice used were C57/Black 6, bred in-house and weighed on average 35 g. Male Sprague–Dawley rats (Charles River, Hungary, Isaszeg) weighing 190–210 g were used after 1 week quarantine in our animal house (accredited according to GLP). The animals were fed with the standard chow diet from the same supplier.

Isolation and culture of mouse Clara cells

Mice were sacrificed by intraperitoneal injection of pentabarbitone. Lungs were perfused with saline, digested with trypsin and Clara cells were isolated as described previously (Masek & Richards 1990). The procedure used here differed from that of Masek and Richards (1990) as follows; gentamycin and anti-PPLO were omitted from all solutions and the medium used throughout the isolation and for culture was a 1 : 1 mixture of Hams F12 (Gibco) and M-199 medium (Gibco) supplemented with 2 mM L-glutamine, 10 μg/ml insulin, 5 μg/ml transferrin, 100 ng/ml hydrocortisone, 10 ng/ml EGF, 0.1 ng/ml retinyl acetate and Pen/Strep (Gibco, 100 U/ml penicillin, 100 μg/ml streptomycin). Once isolated, cells were either cytospun onto glass slides (day 0) or plated onto 16-well glass chamber slides (Gibco) which had been pre-coated with 50 μg/ml fibronectin, and incubated at 37 °C, 5% CO_2. Cells were allowed to attach overnight after which the medium was replaced to remove dead and unattached cells. Medium was subsequently replaced every 2 days.

Isolation and culture of rat type II cells

Rats were deeply anaesthetised with 60 mg/kg pentobarbitone (Nembutal, Sanofi, Paris, France) intraperitoneally, and then killed by severing the abdominal aorta. Type II cells were isolated according to methods described previously (Richards *et al.* 1987, Hoet *et al.* 1994). Cells were cytospun onto glass slides (day 0) or plated onto 24-well plates (Falcon) and incubated at 37 °C, 5% CO_2. Culture medium was DMEM (Sigma) supplemented with 10% FCS (Gibco) and Pen/Strep (Sigma; 100 U/ml penicillin, 100 μg/ml streptomycin). Medium was replaced every 2 days.

Lectin histochemistry

For lectin binding to cultured cells, cytospun cells and plated cells were fixed at appropriate time points by incubating at room temperature in 4% buffered neutral formalin (pH 7.4) for 10 s. The following biotinylated lectin agglutinins (Sigma) were used: *Bauhinia purpurea* (BPA, specificity: β-Gal(1→3)GalNAc), Concanavalin A (ConA, specificity: α-D-Man, α-D-Glc), *Helix pomatia* (HPA, specificity: α-D-GalNAc), *Maclura pomifera* (MPA, specificity: α-D-Gal, α-D-GalNAc), Wheat germ (WGA, specificity: (D-GlcNAc)$_2$, NeuNAc). Lectins were diluted to a final concentration of 20 μg/ml in Tris-buffered saline (TBS), pH 7.4, which contained 10 mM $CaCl_2$, 0.2 mM $MgCl_2$ and 1 mM $MnCl_2$. All incubations were carried out at room temperature. Cells were incubated with lectins for 20 min, rinsed with TBS and incubated for 30 min with either avidin–biotin–alkaline phosphatase (AB–AP) or avidin–biotin–horseradish peroxidase (AB–HRP) (Vector Laboratories). Binding was visualised by staining for 10–15 min with Vector Red or 3,3′-diaminobenzidine (DAB) which are substrates for AP and HRP respectively. As controls for specificity, lectins were pre-incubated overnight at 4 °C with appropriate hapten sugars before incubation with cells (BPA: NAcGal; MPA: D-Gal; HPA: NAcGal; WGA: NAcGlc; ConA: Man + Glc).

For lectin binding to tissue, lungs were fixed in 10% buffered neutral formalin (pH 7.4) for 2 days before routine processing and embedding in Paraplast (Sigma). Sections were cut at 4–5 μm, dewaxed, rehydrated and lectin binding was carried out and visualised as described above.

Immunocytochemistry

For CC10 and keratin immunocytochemistry, cells were fixed respectively in formalin as above and in methanol for 7 min at −20 °C. Cells were incubated with primary antibody for 2 h, rinsed with TBS–0.1% Tween 20, incubated with biotinylated secondary antibody for 30 min, rinsed and binding was visualised as above with AB–AP and Vector Red or Vector Blue substrate. For CC10 binding to lung tissue, tissue sections

were obtained as described above, dewaxed, rehydrated and CC10 immunohistochemistry was carried out as described above for cells. The CC10 antibody was a generous gift from Dr. Gurmukh Singh, Deptartment of Veterans Affairs Medical Center, Pittsburgh, Pennsylvania, USA. The keratin antibody (clone MNF116), a pan-keratin antibody which recognised keratins 5, 6, 8, 17 and 19, was bought from DAKO, UK.

Nitrotetrazolium blue assay

The nitrotetrazolium blue (NBT) solution consisted of 100 µl NBT stock solution (Boehringer Mannheim) in 10 ml 0.1 M Tris pH 9.5, 0.05 M $MgCl_2$, 0.1 M NaCl, 0.1% NADPH. Cells were fixed in formalin as above and incubated with the NBT solution for 10 min at 37 °C. Positive cells stained strongly purple.

Alkaline phosphatase

One tablet of Naphthol AS-TR phosphate (Sigma) was dissolved in 10 ml deionised water and 3 tablets of Fast Red RC (Sigma) were dissolved in 5 ml Tris-buffered saline (pH 8.4–8.6). Cells were incubated in a mixture of 5 ml AS-TR and 5 ml Fast Red for 30 min at room temperature. Positive cells were stained bright red. Red cells with 4 or more lamellar bodies were considered to be type II cells.

Electron microscopy

Day 0 cells were pelleted by centrifugation and cultured cells were harvested by trypsinisation and pelleted. Pellets were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) overnight and post-fixed in buffered 1% osmium tetroxide (0.1 M with respect to sodium cacodylate, and 0.04 M with respect to potassium ferrocyanide) for 2 h. After thorough washing in distilled water, the pellets were stained *en bloc* in 5% aqueous uranyl acetate overnight at 4 °C, followed by dehydration through an alcohol series, and embedding followed by polymerisation in Araldite resin (TAAB Laboratories Ltd., Aldermaston, UK). Ultrathin sections of approximately 90 nm thickness were cut into 300-mesh copper grids, and stained with lead citrate. The sections were examined in a Jeol 100 CX-II transmission electron microscope at an accelerating voltage of 80 kV.

Results

Mouse Clara cells

Lectin binding – lung tissue

BPA, MPA, ConA and WGA reacted strongly with the apical membranes of bronchiolar Clara cells in mouse lung tissue. HPA showed no reactivity. BPA and MPA reactivities are illustrated in Figure 1A and D. Lectin binding was completely blocked or significantly weakened by control hapten treatment.

Lectin binding – cultured cells

The binding activities of the above lectins to mouse Clara cells were examined over 5 days in culture (Table 1). By day 7, cultures had begun to visibly appear necrotic, therefore day 5 was used as the final time point. ConA and WGA both showed very strong binding activity to freshly isolated cells, and all cells were stained. Staining remained strong at day 1 and day 5 in culture. BPA showed no reactivity with freshly isolated cells (Figure 1B) and only a few scattered positive cells (less than 5%) were present at day 1. However, at day 5, 30–40% cells bound BPA (Figure 1C). MPA produced moderately positive staining in freshly isolated cells (Figure 1E) and cultured cells at day 1 and day 5 (Figure 1F) were all strongly positive. HPA showed no reactivity with isolated or cultured cells at any stage.

Electron microscopy

When examined by EM, fresh isolates were found to consist of approximately 78% Clara cells, indicating relatively high levels of purity. The remainder were almost all ciliated cells. Day 1 and day 5 cultures consisted of approximately 80% and 85% Clara cells respectively and again, the remaining cells were almost exclusively ciliated cells. Clara cell viability was high at each time point with less than 25% cells appearing apoptotic or necrotic. However, at day 5, there appeared to be a drop in the mitochondrial size and number per cell compared to day 1.

CC10

CC10 is a protein primarily expressed by bronchiolar Clara cells in rodents and humans (Singh & Katyal 1997). Its function is unclear but it may play a role in regulation of inflammation. CC10 immunoreactivity was demonstrated in the apical membrane regions of bronchiolar cells in mouse lung tissue (Figure 1G). Freshly isolated cells were strongly positive for CC10 immunoreactivity and remained so at day 1 (Figure 1H). Staining appeared stronger within clumps of cells which had not spread fully compared with larger, flattened cells. However, this could be because the unspread cells had a less visible volume than those that had spread, but had an equivalent amount of stain. By day 5 in culture, CC10 immunoreactivity appeared slightly reduced (Figure 1I).

NBT assay

Functional Clara cells contain high levels of NADPH-dependent reductase activity. This can be detected histochemically using an NBT assay in which a colourless NBT solution is converted to a purple formazan product by NADPH-dependent reductase. Brief fixation with formalin prior to staining eliminates the low levels of enzyme activity in other cell types likely to be present. Following staining, approximately 75% freshly isolated cells were dark purple in colour, indicating that these were functional Clara cells (Figure 1J). Most positive cells were present within clumps. The number of NBT- positive cells declined in culture. At day 1 and day 5 (Figure 1K), approximately 50% and 40% respectively stained dark purple. Again, most positive cells were

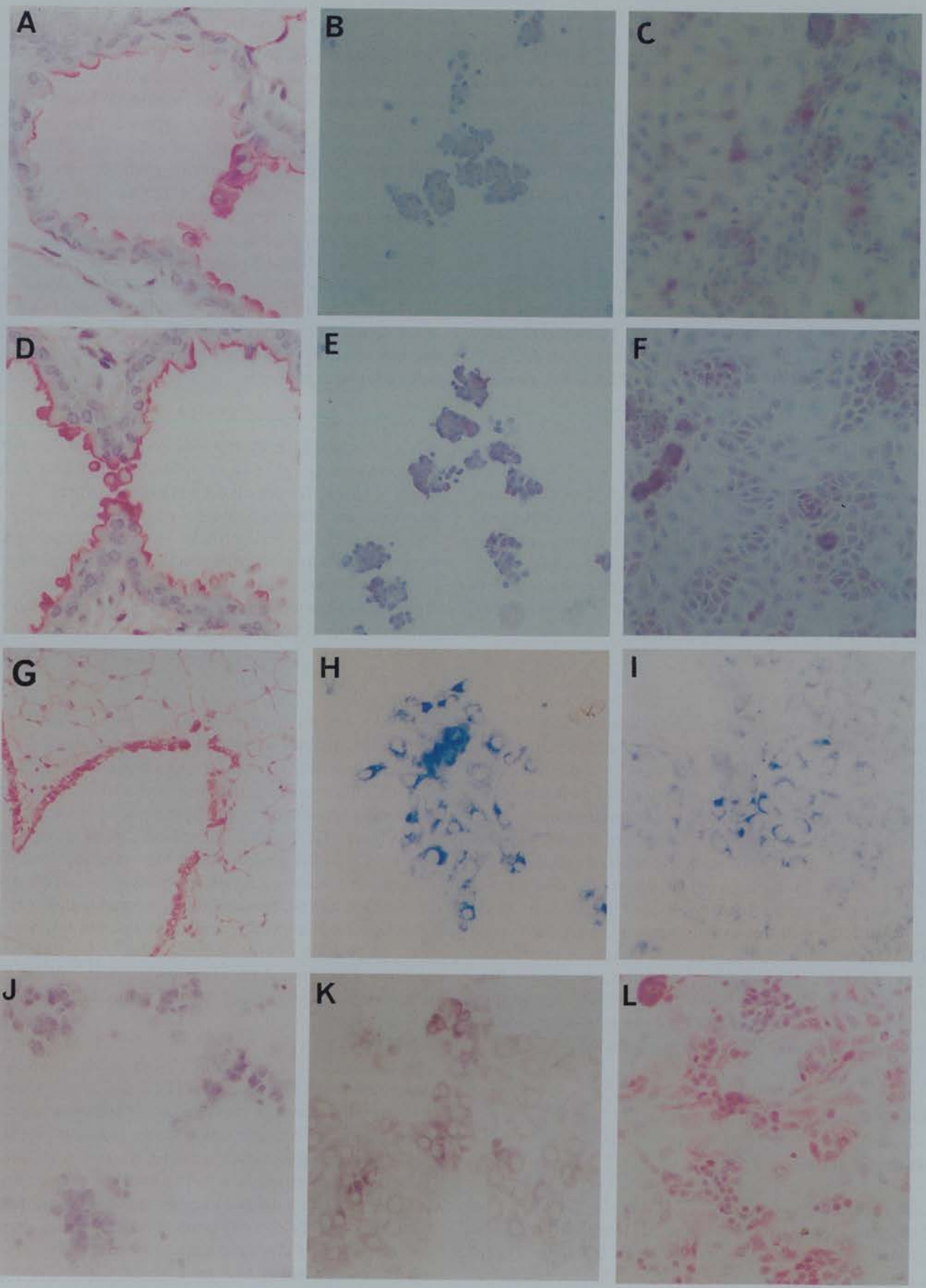

Figure 1. Characterisation of cultured mouse Clara cells. (A) BPA reacted with apical membranes of bronchiolar Clara cells in mouse lung tissue. (B) No BPA reactivity was apparent in freshly isolated (day 0) cells but (C) reactivity was detectable again at day 5 in culture. (D) MPA also reacted with apical membranes of bronchiolar Clara cells in mouse lung tissue. Reactivity was also evident in (E) freshly isolated (day 0) cells and (F) cultured

Table 1. Lectin binding to mouse Clara cells in lung tissue and during culture.

	BPA	ConA	HPA	MPA	WGA	CC10	NBT	Keratin	EM
Lung tissue	++	++++	0	+++	+++	++++	n.d.	n.d.	n.d.
Cells day 0	0	++++	0	++	+++	+++	75%	95%	78%
Cells day 1	+	++++	0	+++	+++	+++	50%	100%	80%
Cells day 5	++	++++	0	+++	+++	++	40%	100%	85%

Note: ++++ = very strongly positive; +++ = strongly positive; ++ = moderately positive; + = weak/occasional positive; 0 = no stain; NBT = nitrotetrazolium blue; EM = electron microscopy; n.d. = not determined; n=3.

present within clumps of cells which had not spread fully, rather than in the areas of flattened, spread cells.

Keratin immunocytochemistry

Approximately 95% freshly isolated cells contained keratin, as detected immunocytochemically, indicating their epithelial origin. At day 1 and day 5 (Figure 1L), all cells were keratin positive suggesting that contaminating non-epithelial cells such as macrophages or fibroblasts present in initial isolates had either failed to attach or survive in culture.

Rat type II cells

Lectin binding – lung tissue

BPA reacted strongly with the surface membranes of type I cells in rat alveolar tissue but there was no reaction in the apical membranes of type II cells (Figure 2A). Very strong reactivity was demonstrated with MPA in the membranes of type II cells whereas type I cells did not show reactivity with MPA (Figure 2C). Lectin binding was completely blocked or significantly weakened by control hapten treatment.

Lectin binding – cultured cells

Lectin binding capacity was studied for 8 days and the results are summarised in Table 2. BPA reacted with freshly isolated type II cells and reactivity remained for the duration of the cultivation. MPA reactivity on day 1 was identical with BPA reactivity (Figure 2B and D). MPA membrane staining could be detected on 90–95% of cells, however, intensity significantly declined by day 4. ConA, HPA and WGA bindings varied during the culture period.

Electron microscopy

The purity of the type II cell cultures was similar to that of the Clara cell cultures up to day 4, with over 70% of cells identifiable by EM as type II, that is, containing at least 4 lamellar bodies. However, by day 6, a dramatic reduction in the number of identifiable type II cells occurred, and by day 8, no cells contained lamellar bodies.

Alkaline phosphatase

There was a progressive loss of alkaline phosphatase activity during the culture period with no detectable activity remaining by day 8 (Table 2).

Discussion

Our understanding of the various differentiation pathways operating in lung epithelium is limited. This is due in part to the apparent ability of lung epithelial cells to differentiate along multiple pathways (Johnson *et al.* 1990) and also the occurrence of various 'intermediate' phenotypes, both *in vivo* and *in vitro*, which have features characteristic of more than one cell type, hindering accurate identification (Jeffery & Li 1997). Demonstration of cell surface glycoproteins using lectin histochemistry has been proposed as a possible method for identifying specific lung cells *in vivo* and *in vitro*, and tracking subsequent routes of differentiation which occur, for example, during normal homeostasis, following injury or during disease, or in culture. However, many of the lectin histochemical studies reported in lung epithelial cells to date are conflicting or have not included alternative methods of cell identification for comparison with lectin receptor expression. This makes interpretation of observations difficult. In this study we have attempted to clarify the lectin binding properties of 2 subpopulations of cultured lung cells and to evaluate the potential of lectins as markers of differentiation in these cells. Mouse Clara cells and rat type II cells were cultured for 5 and 8 days respectively. During this time, their capacity to bind each of a panel of 5 lectins was determined and compared with binding to corresponding cells in mouse and rat lung tissue *in situ*. In addition, parallel biological and EM analyses were carried out to provide a comprehensive evaluation of the relevance of lectin receptor expression to cell differentiation in culture.

At the ultrastuctural level, EM analysis indicated that the mouse Clara cell cultures consisted of approximately 80% Clara cells at each time point, as identified by the presence of high numbers of mitochondria, secretory granules and

cells at day 5. CC10 immunoreactivity was present in bronchiolar Clara cells in (G) mouse lung tissue and (H) cultured cells at day 1. However, (I) immunoreactivity had decreased by day 5 of culture. (J) Approximately 75% freshly isolated Clara cells were NBT positive but (K) positivity had decreased by day 5. (L) All cells present in day 5 cultures were keratin positive. Magnification, A and D: ×400; all others, ×200.

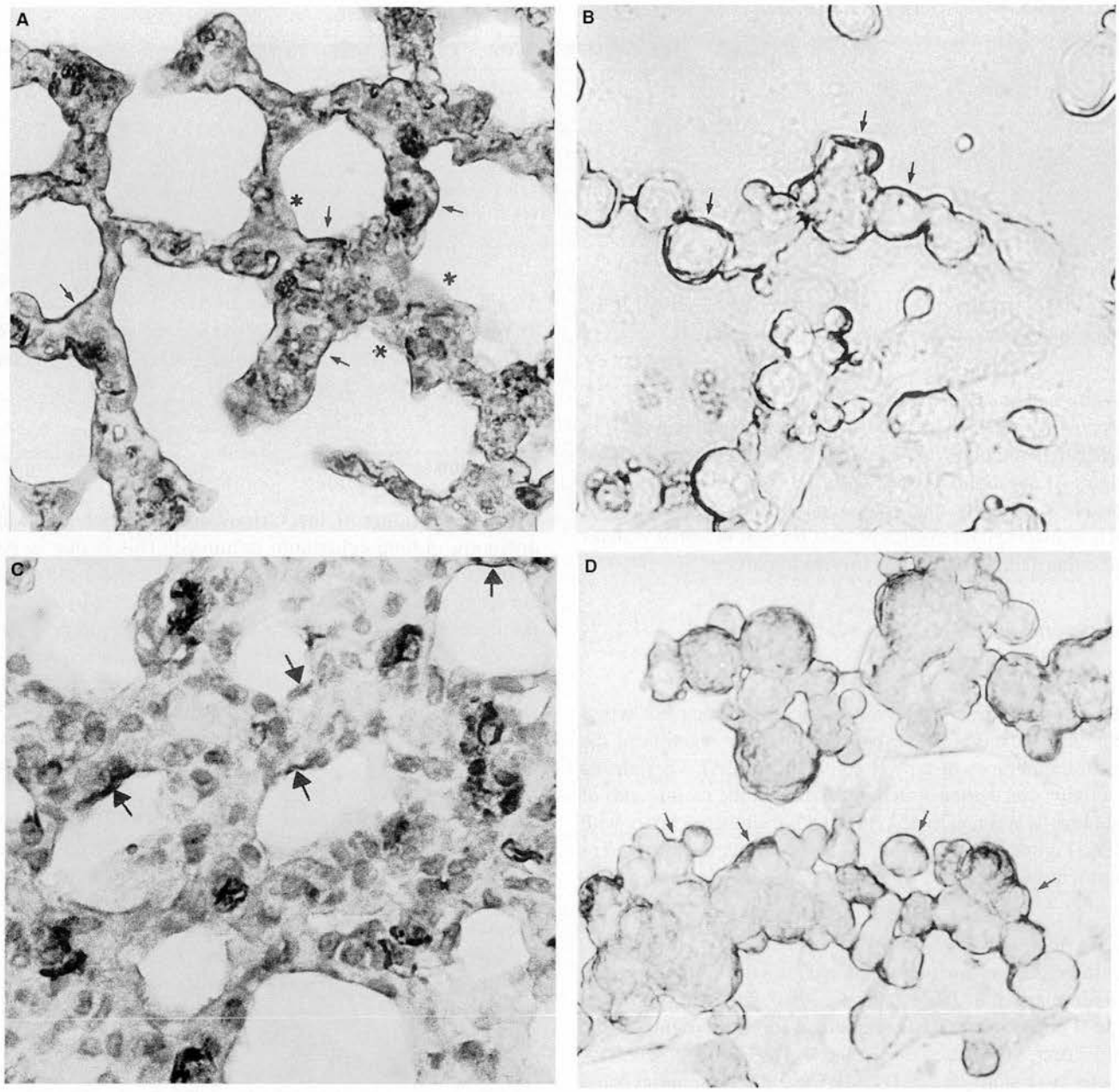

Figure 2. Characterisation of cultured rat type II cells. (A) BPA reacted strongly with apical membranes of alveolar type I cells (arrows) but not type II cells (asterisks) in rat lung tissue. In contrast, (B) BPA reacted with the majority of cultured type II cells (arrows) at day 1. (C) MPA reacted strongly with apical membranes of alveolar type II cells (arrows) in rat lung tissue and (D) reactivity remained at day 1 in culture (arrows). Magnification, A, B and D: ×650; C: ×320.

abundant endoplasmic reticulum. Ciliated cells accounted for almost all of the remaining 20% of cells at each time point. Interestingly, in these EM studies, there was no evidence of Clara cell differentiation over the time in culture. Keratin immunohistochemistry confirmed the lack of contaminating non-epithelial cells in the cultures. However, the decline of NADPH-dependent reductase activity demonstrated by NBT histochemistry and the reduction in CC10 immunoreactivity suggested that the Clara cells were losing at least some of their more specialised functions, a common occurrence in cultured cells. The reactivity of 4 of the 5 lectins (ConA, MPA, WGA all strongly reactive and HPA non- reactive) was identical when examined in Clara cells *in situ* in mouse lung tissue and in Clara cells cultured for 5 days. Only BPA showed altered reactivity *in vitro* compared to *in vivo*. The loss of BPA reactivity at day 0 and its reappearance at day 1 and day 5 in cultured Clara cells suggests that the BPA-reactive glycoprotein was altered or damaged during the isolation procedure, possibly being cleaved during trypsinisation, but was subsequently repaired or resynthesised in culture.

Table 2. Lectin binding to rat type II cells in lung tissue and during culture.

	BPA	ConA	HPA	MPA	WGA	AP	EM
Lung tissue	0	++++	++	++++	+++	n.d.	n.d.
Cells day 0	+++	++++	++	++++	+	85%	80%
Cells day 1	+++	++++	++	++++	+++	81%	85%
Cells day 2	+++	++++	++	++++	++	50%	71%
Cells day 4	+++	+++	++	++	++	42%	70%
Cells day 6	+++	+++	++++	++	+	5%	8%
Cells day 8	+++	+++	+++	++	+++	0%	0%

Note: ++++ = very strongly positive; +++ = strongly positive; ++ = moderately positive; + = weak/occasional positive; 0 = no stain; AP = alkaline phosphatase activity; EM = electron microscopy; n.d. = not determined; $n = 3$.

These findings indicate that at the ultrastuctural and cell surface glycoprotein levels, Clara cells cultured under the conditions described here closely resemble Clara cells *in vivo*. The repertoire of cell surface glycoproteins of cultured Clara cells was very similar to that *in vivo*. Except for the temporary alteration in BPA reactivity, the composition of the culture medium or the culturing process itself appeared to have no effect on the expression of the glycoproteins involved, or alter their chemical composition to render them unrecognisable by the lectins. Despite this, however, specialised functions such as NADPH-dependent reductase activity and CC10 expression declined in culture. While future optimisation of the culture conditions may improve retention of such functions, it appears that ultrastructural or lectin binding studies alone do not provide an accurate reflection of Clara cell function *in vitro*. Biochemical measurements such as NBT reductase activity, CC10 production and cytochrome p450 activity, for example, should be carried out in parallel.

Cultured rat type II cells, in contrast to mouse Clara cells, reacted with HPA although the reactivity was found to vary during the time in culture. As is already known, ConA binding was not specific to type II cells, but also bound to type I cells. The cells showed variable reactivity with WGA also. Cultured type II cells showed altered reactivity for MPA and BPA compared with type II cells *in vivo*. BPA binding capacity, which we found to be characteristic of type I cells but not type II cells *in vivo*, appeared during the type II cell isolation and its intensity did not change during the culture period. The intensity of MPA binding in the cells decreased by day 4. At the same time, alkaline phosphatase activity, a functional parameter of type II cells, rapidly decreased (only 42% of cells were AP positive at day 4), in contrast to EM analysis which indicated that 70% of cells had the characteristic features of type II cells containing 4 or more lamellar bodies. At day 6, EM findings were more consistent with AP activity (5%) with only 8% of cells containing 4 or more lamellar bodies. By day 8, AP activity had ceased and the cells did not exhibit the characteristic ultrastructure of type II cells.

On the basis of the above results, the appearance of BPA positivity appears to be an early sign of altered surface properties. The decrease in AP activity and MPA reactivity are similarly early and sensitive signs of morphological and functional alterations in type II cells, although at this early stage the cell ultrastructure still resembles type II cells. The aetiology of changes in ConA, HPA and WGA bindings was not dealt with in this study.

The stability of BPA binding and the decrease in MPA reactivity and AP activity suggest that in the course of cultivation, intermediate type cells develop. These intermediate cells differ from *in vivo* type II cells, showing some surface properties (MPA positivity) but revealing also stable oligosaccharide sequences characteristic of type I cells.

It is, therefore, proposed that lectin histochemistry combined with enzyme histochemistry may offer a very sensitive and useful tool for monitoring type II cells in culture as well as *in vivo*.

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